BIOCHEMICAL STUDIES ON THE UNCONVENTIONAL RAC SPECIFIC GEF, DOCK180

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BIOCHEMICAL STUDIES ON THE UNCONVENTIONAL RAC SPECIFIC GEF, DOCK180

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The Rho family of GTPases comprises of approximately twenty family members, which regulate multiple cell activities including cellular migration, cell polarity, vesicle trafficking and a variety of enzymatic activities. Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by removal of bound GDP and the subsequent of loading of GTP, which leads to downstream effector recognition. Two families of GEFs have been described: The classical Dbl-GEF family members share conserved DH-PH domains, where the DH domain harbors the GEF activity for the Dbl family members while the PH domain is thought to help localize the GEF protein to the plasma membrane. In contrast to Dbl-GEFs, the more recently described Dock180 GEF-family members do not share primary sequence homology with DH-PH domains, although they exhibit robust nucleotide exchange activity for Rho GTPases. Here we describe the biochemical characterization of the conserved limit DHR-2 domain of Dock180 and its activation of the Rac GTPase. We delineate a limit functional sub-domain of DHR-2 which is composed of approximately 300 residues in the C-terminal portion of DHR-2 (referred to below as DHR-2c). Our data show this region is both necessary and sufficient for robust GEF activity as the DHR-2 domain specifically activated Rac both *in vitro* and *in vivo*. Scanning mutagenesis of Rac also revealed that DHR-2c binds to Rac in a manner distinct from the classical Dbl-family Rac-GEFs. Specifically, both alanine 27 and tryptophan 56 of Rac are demonstrated to provide a bipartite recognition site for DHR-2c GEF-specific recognition, whereas, for

Dbl-family GEFs, tryptophan 56 of Rac is the primary determinant of GTPase specificity. We also identified the corresponding residue (methionine 1524) on Dock180 to specifically recognize tryptophan 56 of Rac. These results define the core residues for Dock180's guanine nucleotide exchange activity while highlighting recognition sites that underline GTPase specificity for Dock180-family members.

BIOGRAPHICAL SKETCH

The author was born on November 27th, 1980 in Changzhou, a small city in middle-east China. He grew up there until he graduate from high school in 1998, because of the curiosity in life science, he enrolled in the department of biological sciences and technology at Tsinghua University in Beijing, China. He finished his thesis in Dr. Zihe Rao's laboratory and got the B.S. degree.

The author was accepted to biophysics field at Cornell University in 2002 and joined Dr Richard Cerione's laboratory soon after. During the six years of work there, he was focusing on the biochemical and structural characterization of multiple proteins involved in signaling pathways and finished his thesis in January 2010. He will return to China and start a pharmaceutical firm with friends and alumni.

ACKNOWLEDGEMENTS

Having spent seven years in my Ph.D studies in Dr. Richard Cerione's laboratory at Cornell, I really learned and achieved a lot. I can never imaging the 'Dr.' title before my name until it does come true. The whole process is filled with failure and success, depression and happiness, self-deny and achievement. I can not count how many people have helped me in the past seven years. Here, I sincerely want to express my appreciation to you all.

Dr. Rickard Cerione, my advisor, has given me support and help in all my Ph.D. studies. When I was first admitted to this lab, I was a fresh graduate student and the knowledge about life science is very shallow. Under his mentoring, I became a real biologist step by step. I hope I can always have that enthusiasm in research which Rick always has.

I want to thank Dr Jon Erickson for his direct mentoring. He is always very patient to help me with my experiments design and discuss the problem with me whenever I was stuck. I can always learn a lot after talked to Jon.

All the lab members in the Cerione' group are always very friendly and helpful. It is my big honor to work in such a warm laboratory and I can get a lot from the lab members besides the research. I will miss here because this is really like a big family for me in U.S.

No words can express my appreciation to you, my beloved wife Ling. You are with me any time, any place. Now we can start our new careers together and fight for best future. Also, I want to thank my parents who are always supportive and my daughter who can always make me happy.

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INTRODUCTION

1.1 Rho GTPases and their regulators

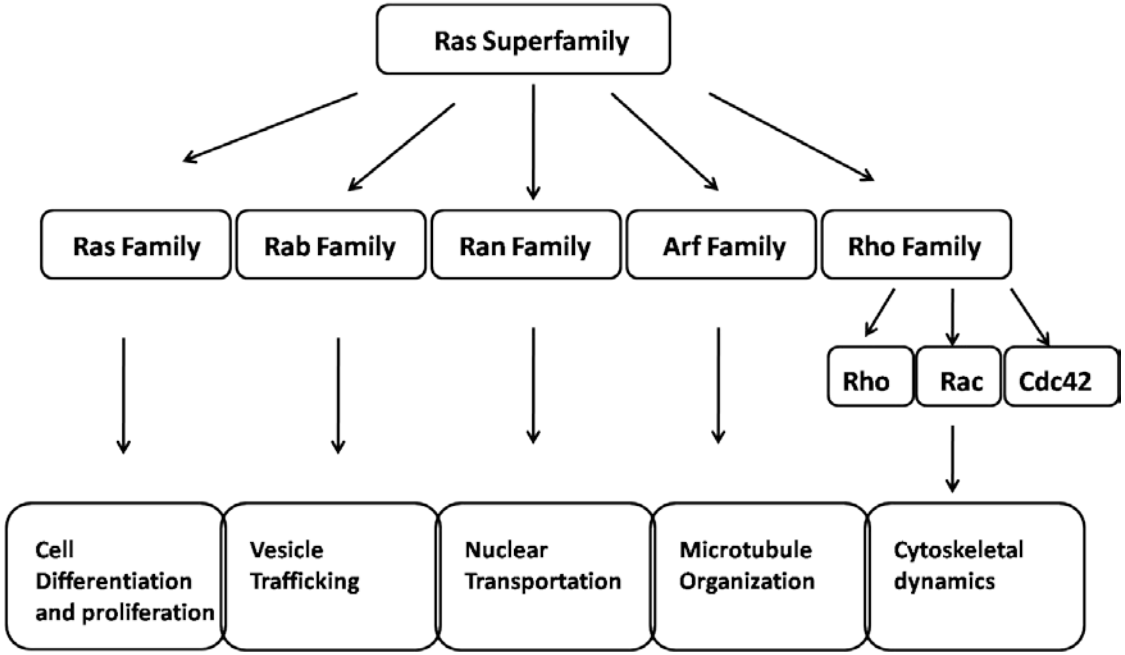
1.1.1 GTPase cycle

The Ras GTPase superfamily comprises a large number of proteins, which participate in a wide range of signaling pathways in cells. They are key regulators of a number of biological processes including cell movement, cell differentiation, programmed cell death, vesicle transport and the regulation of the actin cytoskeleton (*1*, *2*). Based on their structural and functional distinctions, this superfamily can be further divided into five families referred to as the Ras, Rab, Ran, Arf and Rho subfamilies (*3*). As outlined in Figure 1.1, Ras family members are generally thought to control cell proliferation(*4*) while Rab GTPases control vesicle trafficking (*5*). Rho GTPases direct cell mobility and cytoskeletal reorganization and stimulate the activation of specific nuclear kinases (*6*) while Ran GTPases regulate nuclear import and export(*7*), and Arf GTPases influence intracellular trafficking and microtubule organization (*8*). However, the specific functions of Ras superfamily proteins are not limited to the above mentioned processes but in fact can impact a broad range of cellular events. Members of different families may also cooperate in specific signaling pathways (*9*).

The Rho (for Ras-homologous) family of GTPases includes of approximate twenty members (*10*). The best studied members are Rho, Rac and Cdc42. Like all other GTP-binding proteins, Rho family proteins exist in two different states, a GDP-bound inactive state and a GTP-bound active state. The GTP-binding/GTP-

Figure 1.1 Schematic representation of the five sub-families of the Ras superfamily and their cellular functions

Small GTPases



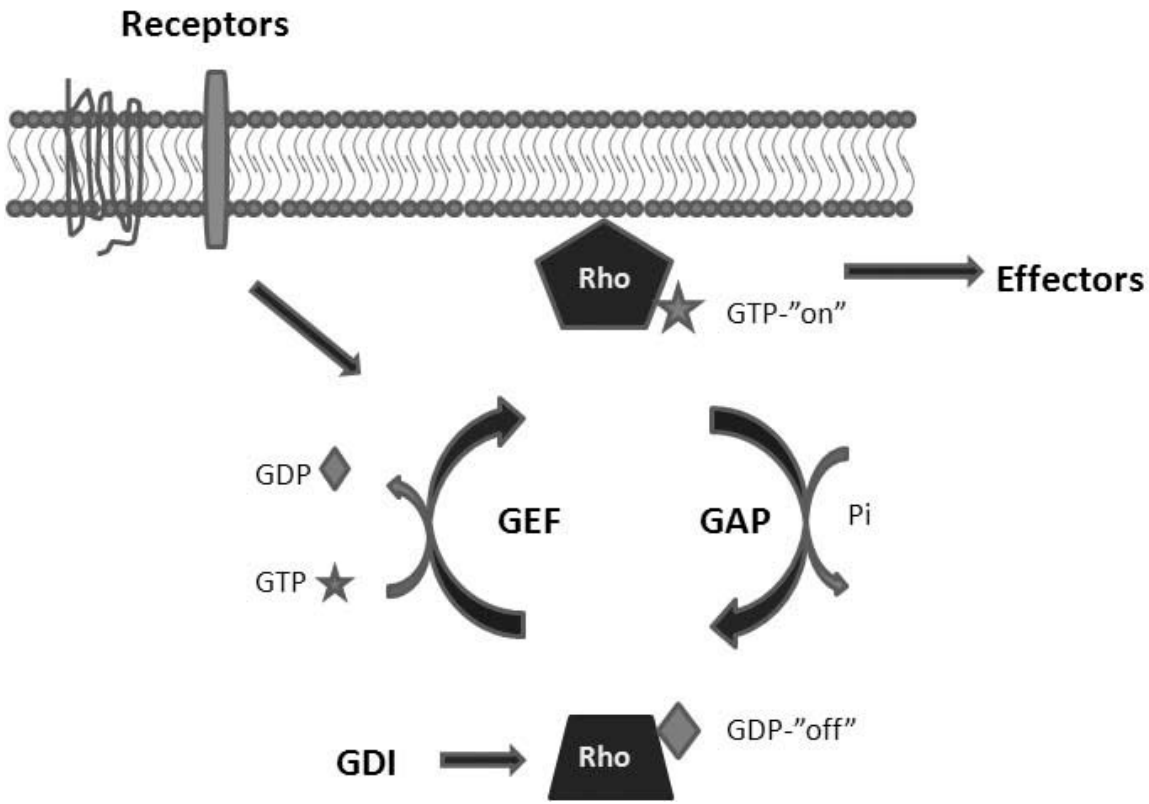
hydrolytic cycles of Rho proteins are tightly regulated as their intrinsicrates of GDP-GTP exchange and GTP hydrolysis are very low and not sufficient to quickly respond to cellular signals. With the help from two families of regulatory proteins, GEFs and GAPs, as summarized in Figure 1.2, Rho GTPases can be turned on and off at much higher rates. GEFs (for guanine nucleotide exchange factors) stimulate the exchange of GDP for GTP on Rho GTPases (*11*) while GAPs (for GTPase-activating proteins) accelerate the hydrolysis of GTP to GDP and thereby shutdown the function of the GTPases (*12*).

1.1.2 Signaling from receptors to Rho GTPases to biological effectors

In 1992, the first cellular response to Rho protein activation was discovered (*13*). Since then, a number of cellular signaling activities and biological responses involving Rho GTPases have been identified. A good deal of progress has also been made regarding the identification of extracellular stimuli and cell surface receptors that promote the activation of Rho proteins. For example, Cdc42 has been shown to be activated by several types of extracellular signals. One in particular is EGF, which binds to the EGF receptor tyrosine kinase. The EGF-dependent activation of Cdc42 is mediated through the activation of the Src tyrosine kinase and various Cdc42 GEFs including Vav2 and Cool-1 (*14*, *15*). RhoA can be activated by LPA, which binds to a G protein-coupled receptor and activates the large G protein $G_{12/13}$. Activated $G_{12/13}$ in turn binds to the RGS (for regulation of G protein-signaling) domain of a Rho-GEF (p115-RhoGEF) which activates RhoA (*16*).

Activated Rho family GTPases bind to a wide variety of downstream targets and effector proteins. Among the original functions discovered for the Rho-family

Figure 1.2 Signaling from receptors to Rho GTPases to effectors. The GTPase cycle is regulated by GEFs, GAPs and GDIs.


Receptors
Rho
GTP-"on"
Effectors
GDP
GTP
GEF
GAP
Pi
GDI
Rho
GDP-"off"

GTPases is the regulation of actin polymerization. Specifically, Rho proteins contribute to contractility and actin stress fiber formation. Rac stimulates the formation of lamellipodia while Cdc42 promotes the formation of filopodia (*13*). There are more than 60 known target and effector proteins that bind to activated (GTP-bound) Cdc42, Rho and Rac. These target proteins generally do not contain similar recognizable motifs. However, most appear to contain a conserved GTPase-binding domain (GBD) which can recognize the GTP-bound form of the Rho GTPases. In some cases, activated Cdc42 regulates the function of effector proteins, i.e. PAK and WASP, by altering the structure of the GBD and the release of an auto-inhibitory (*17*).

Signaling pathways induced by different Rho proteins show a significant degree of crosstalk, as different Rho proteins may activate or inhibit each other either directly or indirectly. For example, Ridley and Hall showed (*13*) that Ras- induced membrane ruffling was due to the Ras-promoted activation of Rac and then RhoA. Cdc42 was later found to be in the same pathway in some cells. The Rho GTPases can also affect other Ras superfamily members either by stimulating a GAP to suppress their activity or by simulating a GEF to activate their signaling function.

1.1.3 Regulating Rho GTPases

Rho GTPases are central participants in cellular signaling pathways. They influence a wide range of cellular and biological responses. It often becomes important that these GTPases are rapidly activated when it is necessary to trigger a cellular response, and then quickly deactivated when the response needs to be terminated. To

achieve this, three main families of regulatory protein are involved, i.e., GEFs, GAPs and GDIs.

To activate Rho GTPases, the rate limiting step is the exchange of GDP to GTP. The function of GEFs is to accelerate the nucleotide exchange reaction. The GEF proteins bind to the GTPases and modify their nucleotide-binding sites, thus reducing the nucleotide- binding affinity. Because of the high concentration of GTP (i.e. 10 fold) in cells, following GEF-catalyzed GDP dissociation, GTP will bind to the GTPase and catalyze its dissociation from the GEF, thus enabling it to bind to its biological effectors (*11, 18*)

The GAP family comprises a group of proteins which help accelerate the rates at which GTPases hydrolyze GTP to GDP. Most of the GAPs for the Rho GTPases share a conserved arginine residue called an 'arginine finger'. It 'points' into the active site of Rho GTPases thereby stabilizing the transition state for GTP hydrolysis. This interaction increases the rate of GTP hydrolysis by more than one-thousand-fold (*19, 20*).

Compared to GEFs and GAPs, the Rho GDI family is much smaller. It contains only three members and a few other proteins which have been suggested to have GDI activity. The GDIs inhibit GDP-dissociation and nucleotide exchange, as well as blocks GTP hydrolysis (*21, 22*). However, the main cellular function of the GDI is to help maintain Cdc42 and other Rho proteins in a soluble state until the appropriate signals are received, enabling the GTPases to bind to effector proteins at the membrane (*23, 24*).

1.2 Dbl-GEFs and Dock180 GEF family

1.2.1 Classic GEF family – Dbl Family

Dbl, which was isolated in 1985 (*25*), was the first Rho GEF to be identified (*26*). It contains a region which showed significant similarity to Cdc24 (*27*), a protein that was suspected to be an upstream activator of Cdc42 in yeast. This region was named the DH-domain (Dbl-homology domain), and is essential for the GEF activity of Dbl(*28*). Since that initial discovery, a number of Rho GEFs have been isolated. There are now known to be 46 Rho GEFs in humans and a number of family members in other species. These proteins comprise what is called the Dbl-GEF family. All of the family members share two conserved domains, a 200-amino-acid DH domain followed by a 100 amino-acid PH-domain (Pleckstrin-homology domain). The DH domain is mainly responsible for the GEF catalytic activity. The PH domain, adjacent and C-terminal to the DH domain, may have several different functions. A primary function is the binding of phosphoinositide lipids as well as other proteins, which in turn can either directly affect the catalytic activity of the DH domain (*29*) or help target the GEF to the appropriate location in cells (*30*). In addition to these two conserved domains, Dbl GEFs may contain other regulatory and binding domains. These domains help to regulate the activity of GEFs or couple the GEFs to upstream receptors or signaling molecules. Some of the GEFs are found to be highly specific for one GTPase, such as Tiam1 for Rac, Intersectin for Cdc42, and p115RhoGEF for Rho. Others may activate several GTPases, i.e., Vav activates Cdc42, Rac and Rho, and Dbl activates Rho and Cdc42 (*31*). The specificity is determined by the structure of the GEFs and GTPases. However, the specific regions on the GEF critical for Rho GTPase recognition still remain to be established.

1.2.2 Regulation of Dbl-GEFs

GEFs are key regulatory proteins as they are directly responsible for activating small GTPases. Mutations in GEFs that cause them to be de-regulated or excessively active give rise to serious diseases including cancer and developmental disorders.

In normal cells, GEF proteins are tightly regulated through a number of mechanisms. One of the most common regulatory mechanisms is auto-inhibition. Vav provides one of the best-understood examples of auto-inhibition. The N-terminal region of Vav bends over and binds to the active site of the DH domain thus blocking the access of Rho-GTPases (*32*). Removal of the N-terminal sequence leads to constitutive activation. The auto-inhibition can be released in certain circumstances. For example, phosphorylation of tyrosine residue 174 of Vav at the N-terminal region releases it from binding to the DH domain (*32*).

The activity of GEFs can also be regulated by direct protein-protein interactions. Cool-2 shows different GEF capability depending upon whether it is monomer or dimer. When Cool-2 is a dimer, it acts as a Rac-specific GEF, whereas when Cool-2 is a monomer, it can serve as a GEF for either Cdc42 or Rac. However, monomeric Cool-2 fails to show any GEF activity unless the SH-3 domain interacts with the Cdc42/Rac –effector protein PAK. PAK-bound monomeric Cool-2 exhibits GEF activity toward both Rac and Cdc42 (*33*). More recently, it was shown that the Rac-GEF activity of dimeric Cool-2 is activated by GTP-bound Cdc42 (*34*).

The GEFs can also be regulated by localization. Because the activation of GTPases by GEFs normally occurs at the plasma membrane, the recruitment of GEFs to the membrane is often an essential step for signaling in cells. The PH domain of GEFs is normally the domain responsible for the localization of the Dbl-GEFs, in many cases as an outcome of its binding to phosphoinositides (*30*).

1.2.3 Dock180 family: Newly discovered Rho GEFs

In 1996, Matsuda's group discovered a protein with a relative molecular weight of 180 kDa, which interacts with the small adapter protein Crk (*35, 36*). Subsequent studies showed that Dock180 and its homologues in *Drosophila* (Myoblast City) (*37*) and *C.elegans* (Ced-5) (*38*) are involved in a number of biological process including actin cytoskeleton reorganization, phagocytosis of dead cells, cell migration and myoblast fusion. Dock180 was shown to be an upstream regulator of Rac, and biochemical analysis confirmed that it functioned as a Rac-GEF. However, Dock180 does not have tandem DH and PH domains and it shares very low sequence similarity with Dbl-GEFs (*39, 40*).

Further examination of gene data bases demonstrated the existence of several related proteins in mammals. Together with Dock180, these proteins form a new GEF family called the Dock180 superfamily (*39*). At least eleven mammalian members and several more in other species, such as worms, fruit flies and yeast, have been discovered. The eleven members have been designated Dock1 (Dock180) to Dock11 for convenience.

Most of the Dock180 family members have been cloned and shown to exhibit GEF activity toward Rho family GTPases. The eleven members have been further divided into four subfamilies named Dock A to Dock D. Within the whole superfamily, there are two domains conserved, which are named the DHR-1 (Dock180 Homology Region-1) domain and the DHR-2 domain. Outside these two domains, different subfamily members have several other domains and the similarity of these regions among different subfamilies is quite low (*40*).

1.2.4 Functions of Dock180 family members

Recently, several groups have been studying the functions of Dock180 and other family members and some characterizations have already been reported. However, in many cases, the mode of action of Dock180 family members is still far from fully understood, especially when compared to what is known about Dbl-GEFs.

The Dock A subfamily includes Dock180 (Dock1), Dock2 and Dock5, with all of the family members being Rac-specific GEFs. Each contains an SH-3 domain at the N-terminal, in addition to DHR-1 and DHR-2 domains. Dock180 has an extra proline-rich domain at its C-terminal end. The SH-3 domain interacts with a protein called Elmo which is essential for the activation for Dock180 and Dock2. The proline-rich domain binds to Crk II, which is a well-known signaling adapter protein (*41*). The detailed mechanism underlying the GEF activity of Dock180 will be elaborated upon later.

Dock2 was found to be important for lymphocyte migration. Chemotaxis is crucial in the immune response. In order to undergo chemotaxis, cells need to establish a polarized morphology, which includes the formation of a leading lamellipodium and a rear compartment. The formation of lamellipodium requires the activation of Rac, which can be promoted by Dock2 during lymphocyte migration. Deletion of Dock2 in mouse cells abolished lymphocyte migration in response to lymphoid chemokines (*42*). However, how Dock2 responds to chemokine receptors is still not understood. Dock5 has been reported to be functional in the establishment of lens integrity but the mechanism is very poorly understood.

The Dock B subfamily includes Dock3 and Dock4. Both are also specific GEFs for Rac similar to the Dock A members. However, the similarity of the DHR-2 domain between the two subfamilies is only approximately 17%. Dock3, also known as MOCA (the Modifier of Cell Adhesion), was originally identified as a presenilin-

binding protein. It has an SH-3 domain and two proline-rich domains. It is localized specifically in neurons and has been demonstrated to bind Rac and induce GTP loading onto Rac, which leads to the activation of JNK and cell morphology changes (*43*). Dock4 was originally discovered as a gene missing in tumor progression. Subsequent research demonstrated that Dock4 has tumor suppressor properties. It is regulated by the small GTPase RhoG and forms a complex with Elmo to activate Rac, which then promotes cell migration. It has also been shown to regulate dendritic growth and branching through activation of Rac in neurons (*44-46*).

The Dock C subfamily is comprised of three members, Dock6, Dock7 and Dock8. These GEFs are different from other Dock180 family members because they are potential GEFs for both Cdc42 and Rac. Dock6 is involved in neurite outgrowth and a Dock8 mutant was found in lung cancer cells (*47, 48*). Both of these proteins are important regulators for lamellipodia formation. Dock7 is the best known member of this subfamily. Recent work has shown that Dock7 is directly activated by the neuregulin/heregulin receptor Erb2. Erb2 binds to Dock7 and phosphorylates Tyr-1118 of Dock7. Activated Dock7 then induces the migration of Schwann cells(*49*). Other studies have shown that Dock7 contributes to the polarization of neurons and to the regulation of axon formation (*50, 51*).

Dock9 (Zizimin1), Dock10 (Zizimin3) and Dock11 (Zizimin2) comprise the Dock D (Zizimin) subfamily. Each of these proteins acts as specific GEFs for Cdc42 (*52*). They share similar primary sequence and function in cells. However, their tissue distribution is distinct. For example, Zizimin1 is enriched in non-hematopoietic tissues, while Zizimin2 is expressed mainly in lymphocytes (*53*). Zizimin2 was originally discovered in our laboratory through its ability to bind to the activated form of Cdc42 (also called ACG – Activated Cdc42-associated GEF). ACG can bind to both the GDP- and GTP-bound forms of Cdc42 which is quite different from other Dock180

GEFs. It binds to activated Cdc42 in a manner that enables the positive feedback stimulation of GDP-GTP exchange on Cdc42, similar to the activation of Cool-2 by GTP-bound Cdc42 (*34*, *54*).

1.3 Function of Dock180 in phagocytosis

1.3.1 Recognizing the apoptotic signals of dying cells

Dock180 is involved in multiple, fundamentally important biological processes which include: actin cytoskeletal changes, cell migration and polarity, and the phagocytosis of apoptotic cells in mammals. Among these various processes, the role of Dock180 in phagocytosis is probably the best understood.

Apoptosis is a form of programmed cell death in multi-cellular organisms. Apoptosis can occur during cell damage, viral infection or as an outcome of disease. It also occurs during negative selection in development or the homeostatic turnover of cells in various tissues. Proper removal of these cells can prevent the leakage of cell contents, secondary necrosis and inflammation. Engulfment also functions in tissue remodeling and immune response regulation. The engulfment of apoptotic cells is mainly executed by those ‘scavenger’ cells, called phagocytes, including macrophages and immature dendritic cells.

Programmed cell death is a very complex process involving multiple signal transduction pathways. It is normally composed of two distinct processes: cell suicide and the removal of dead cells (*55*). To remove apoptotic cells, the dying cells must first be recognized by phagocytes. These signals include the exposure of phosphatidylserine (PtdSer), which is normally located at the inner-membrane of cells (*56*), changes in glycoproteins and lipids on the surface membrane (*57*), or the binding

of thrombospondin to the apoptotic-cell surface (*58*). There are also other poorly-defined molecules which function as the surface signals or 'bridge' signals. To recognize these signals, phagocytes express different types of receptors. For example, PtdSer needs to be recognized directly or indirectly by phagocyte receptors. PtdSer can directly bind to receptors such as BAI1 (*59*), whereas it can indirectly bind to bridging molecules such as calreticulin and C1q and then be further recognized by additional receptors (*60*).

1.3.2 Regulation of actin cytoskeletal remodeling during engulfment in *C.elegans*

After phagocytes receive the signals sent by apoptotic cell, the signals are transferred downstream, causing the cytoskeleton of the phagocyte to reform and surround the apoptotic cell through a complex sequence of events. Dock180-mediated Rac activation in phagocytosis is conserved from *C.elegans*, to *Drosophila* and ultimately to mammals. To understand the mechanism of phagocytosis, researchers have been focusing on the engulfment process in *C.elegans*, a simple model organism, and trying to identify genes involved in how these proteins transfer the necessary signals for engulfment. These efforts have helped to establish a signing network comprised of receptors, activators, inhibitors and effectors. Understanding the network operating in *C.elegans* that is responsible for engulfment should help to identify similar genes in mammals that are involved in similar functions.

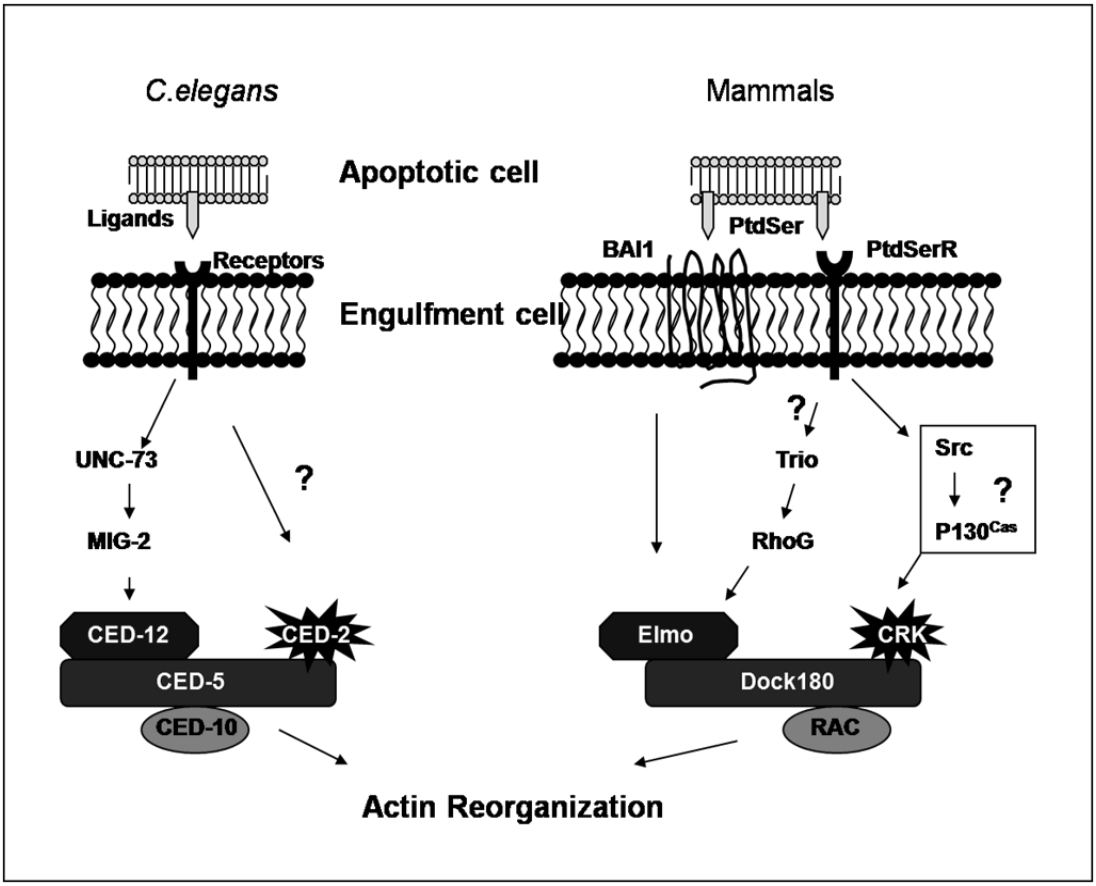
In *C.elegans*, Ced-10 (Rac1 in human) is a key regulator of the actin cytoskeleton during the engulfment of apoptotic cells (*61*). It appears to sit at the crossroad for different signaling pathways involved in apoptosis. Until now, two

independent but partially redundant pathways have been identified that function upstream of Ced-10 (*60*). The first pathway, which is not well studied, consists of Ced1, Ced6 and Ced7. Ced1 and Ced6 function as a receptor complex to recognize signals sent by dying cells (*62*). Their mammalian orthologues are the transmembrane receptor LRP1/MEGF-10 and the adaptor protein GULP. In mammals, LRP1 also interacts with GULP to play a role in phagocytosis. They bind to calreticulin and indirectly interact with PtdSer (*63*). Ced-7 is a unique gene product which is thought to function as a transport protein in both phagocytic and apoptotic cells. However, what it transports and how Ced-7 cooperates with Ced1 and Ced6 remains to be determined. Its orthologue in mammals is ABCA (ATPase Binding Cassette A), which also participates in cholesterol transport across the plasma membrane. The proteins working downstream from Ced1-Ced6-Ced7 and finally activating Ced10 (Rac1) are still not known. However, mutants of Ced1 or Ced6 severely impair phagocytosis, which demonstrates that this pathway is upstream of Rac and critical for engulfment.

The second pathway that functions upstream of Ced-10 includes Ced-2, Ced-5 and Ced-12. Ced-5 is similar to the human protein Dock180 and the *Drosophila melanogaster* protein Myoblast City (MBC) (*37, 38, 64*). It was found to regulate Ced-10 (Rac) (Figure 1.3). Ced-5 mutants are defective in the engulfment of cell corpses. Ced-2 (CrkII in human) interacts directly with Ced-5, which may interact with the receptor and co-localize the Ced-5/Ced-10 complex to the plasma membrane. Ced-12 (Elmo) lacks catalytic activity, but is necessary for the activation of Ced-5. The Ced-2/Ced5/Ced-12 complex is required for Ced-10 activation. However, which receptor is involved in this pathway is still unclear.

In mammalian cells, Rac is linked to actin cytoskeletal reorganization in many types of cellular functions. When Rac is in its active state, it binds to a number of downstream targets which regulate a variety of cellular responses including cell

Figure 1.3 Conserved Rac activation pathway involved with Elmo/Dock180 in both mammals and *C.elegans*


C.elegans
Mammals
Apoptotic cell
Ligands
Receptors
Engulfment cell
PtdSer
BAI1
PtdSerR
UNC-73
?
MIG-2
?
Trio
Src
?
P130Cas
RhoG
CED-12
CED-2
CED-5
CED-10
Elmo
CRK
Dock180
RAC
Actin Reorganization

migration, lamellipodia formation, MAP kinase activation and cell-cycle progression. The mechanism by which Rac is activated is conserved in a number of species, i.e. worms, flies and mammals, and is crucial for apoptotic cell clearance (*38*). Within the Dock180 superfamily, Dock180 is the best known GEF for Rac. *In vivo* experiments have already demonstrated that Dock180 is co-localized with Rac in the membrane ruffles of phagocytes. Deletion of the gene encoding Dock180 severely impairs the uptake of apoptotic cells by phagocytes.

1.4 Regulation of Dock180-Rac complex

1.4.1 The bipartite GEF model with Dock180 and Elmo

It has already been demonstrated that the DHR-2 domain of Dock180 is sufficient to activate Rac both *in vitro* and *in vivo* (*39, 40*). The DHR-2 domain is continuously active in cells and can replace the function of Dock180 in mediating cell elongation and migration. However, in cells, full-length Dock180 is in an inactive state until upstream signals activate its Rac-GEF activity. In the absence of such regulation, it might be expected that Dock180 would give rise to an excessive activation of Rac, which in turn could have important consequences with regard to cancer and other disorders. Thus, an important question concerns how Dock180 is regulated in cells. Several mechanisms have been recently uncovered.

Elmo was the first protein that was found to affect Dock180 function and Rac activation. Elmo does not have any enzymatic activity and can not trigger Rac activation in cells in the absence of Dock180 (*64*). However, the co-expression of Elmo and Dock180 in cells significantly increases GTP-loading onto Rac (*40*). There are three different isoforms of Elmo, designated as Elmo1, Elmo2 and Elmo3. Each can form a complex with Dock180 and several other members of the Dock180

superfamily. The Elmo proteins are comprised of approximately 700 amino acids. They appear to function as scaffold proteins, containing a single PH domain at the C-terminal end followed by a proline-rich motif. In addition, several Armadillo (ARM) repeats are present at the N-terminus which may be involved in binding to the small GTPase RhoG (see below) (*65*). The interaction of RhoG with Elmo may help Elmo to release the auto-inhibition of Dock180.

The auto-inhibition of Dock180 is thought to occur as an outcome of an interaction between its SH-3 domain, located near the N-terminus, and the DHR-2 domain, although there is no obvious proline-rich motif within DHR-2 (*66*). This interaction blocks the binding domain for Rac and thereby prevents Rac activation. Deletion of the SH-3 domain significantly enhances Rac activation in cells by Dock180. Thus, the function of Elmo is to release the auto-inhibition by binding to the SH-3 domain of Dock180. Single residue mutations at the SH-3 domain of Dock180, which abolishes its ability to bind to Elmo, significantly decrease Rac activation by Dock180 in cells. These results have led to the suggestion that Elmo and Dock180 act as a bipartite GEF for Rac activation (*67*).

Several other functions of Elmo were also discovered recently. First, Elmo may help stabilize the transition state for the activation of Rac by Dock180. This was suggested by the finding that in the absence of Rac, Elmo can not bind to a Dock180 mutant lacking the SH3 domain, but it can form a trimeric complex with this Dock180 mutant and Rac (*66*). In addition, the PH domain of Elmo functions to co-localize the Dock180-Elmo complex to the plasma membrane where Rac can become activated and then bind to its effector proteins (*68*). There is also evidence that over-expression of Elmo prevents the ubiquitination of Dock180. Knock-downs of Elmo by siRNAs cause the rapid degradation of endogenous Dock180 (*69*). Interestingly, Crk, which is

another binding partner for Dock180, increases Dock180 ubiquitination when over-expressed.

1.4.2 The Phosphatidylserine Receptor and RhoG activate the Elmo-Dock180 complex

In *C.elegans*, both Ced-5 and Ced-12 have been shown to interact directly with the PtdSer receptor. Recently, in mammalian cells, one of the PtdSer receptors called BAI1 was found to form a ternary complex with Elmo and Dock180 (*59*). BAI1 is a seven transmembrane protein belonging to the family of G-protein-coupled receptors whose extracellular domain binds to phosphatidyserine on apoptotic cells. Inhibition of the expression of BAI1 weakens the engulfment of apoptotic targets. The function of this receptor is not well studied; however, this is the only receptor which has been discovered to directly interact with the Elmo-Dock180 complex.

Other proteins have also been found to be important for the Dock180-mediated activation of Rac through Elmo. As alluded to above, one such protein is RhoG. RhoG recruits the Elmo-Dock180 complex to the plasma membrane (*65*). Also in *C.elegans*, Mig-2, the homologue of RhoG, and its GEF UNC-73, function as upstream regulators of Ced-5-mediated activation of Ced-10 (*70*). However, the identity of the receptors that activate RhoG and how the signal is transferred from the receptors to RhoG remains to be understood.

1.4.3 A parallel signaling pathway to activate Dock180 through Crk II

Although Dock180 was originally discovered as a binding protein for Crk II, there is no definitive evidence that Crk II is essential for the Elmo-Dock180-mediated activation of Rac in cells. The proline-rich-domain truncated mutation of Dock180, which no longer binds to Crk II, can still activate Rac *in vivo* with the help of Elmo. However, recent studies suggest that there is another signaling pathway through Crk II to Dock180, which is possibly parallel to the Elmo-Dock180 pathway (Figure 1.3). It has been reported that integrins, which respond to fibronectin, stimulate $p130^{Cas}$ and paxillin by phosphorylation. Phosphorylated $p130^{Cas}$ binds to Crk II and later forms a complex with Dock180 (*41, 71*). While this pathway offers some intriguing possibilities regarding the cellular regulation of Dock180, its true cellular and biological significance is still not known.

1.4.4 The importance of The DHR-2 domain

To understand the mechanism of Rac activation by Dock180, the DHR-2 domain needs to be investigated in detail. Thus far, not much work has been done in the way of biochemical characterization of this domain. The DHR-2 domain of Dock180 as well as other family members is difficult to express in *E.coli*, and consequently, recombinant preparations of high purity and quality protein have been extremely difficult to obtain. However, in our laboratory, we have successfully cloned a functional domain of DHR-2 and expressed this domain in *E.coli*. This has opened the way to a good deal of biochemical studies and mutagenesis analysis. In the next two chapters, I will focus on the discovery of a new limit functional region for DHR-2, my efforts to obtain its structure, and biochemical studies comparing the mechanism used DHR-2 versus the DH domain of Dbl-GEFs to activate Rac.

REFERENCES

1. Chardin, P. (1988) The ras superfamily proteins, *Biochimie 70*, 865-868.
2. Evans, T., Hart, M. J., and Cerione, R. A. (1991) The Ras superfamilies: regulatory proteins and post-translational modifications, *Curr Opin Cell Biol 3*, 185-191.
3. Etienne-Manneville, S., and Hall, A. (2002) Rho GTPases in cell biology, *Nature 420*, 629-635.
4. Feig, L. A., and Cooper, G. M. (1988) Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP, *Mol Cell Biol 8*, 3235-3243.
5. Novick, P., and Brennwald, P. (1993) Friends and family: the role of the Rab GTPases in vesicular traffic, *Cell 75*, 597-601.
6. Erickson, J. W., and Cerione, R. A. (2001) Multiple roles for Cdc42 in cell regulation, *Curr Opin Cell Biol 13*, 153-157.
7. Melchior, F., Paschal, B., Evans, J., and Gerace, L. (1993) Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor, *J Cell Biol 123*, 1649-1659.
8. Kahn, R. A., Volpicelli-Daley, L., Bowzard, B., Shrivastava-Ranjan, P., Li, Y., Zhou, C., and Cunningham, L. (2005) Arf family GTPases: roles in membrane traffic and microtubule dynamics, *Biochem Soc Trans 33*, 1269-1272.
9. Burridge, K., and Wennerberg, K. (2004) Rho and Rac take center stage, *Cell 116*, 167-179.
10. Ridley, A. J. (2001) Rho family proteins: coordinating cell responses, *Trends Cell Biol 11*, 471-477.

11. Erickson, J. W., and Cerione, R. A. (2004) Structural elements, mechanism, and evolutionary convergence of Rho protein-guanine nucleotide exchange factor complexes, *Biochemistry 43*, 837-842.
12. Bos, J. L., Rehmann, H., and Wittinghofer, A. (2007) GEFs and GAPs: critical elements in the control of small G proteins, *Cell 129*, 865-877.
13. Ridley, A. J., and Hall, A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors, *Cell 70*, 389-399.
14. Feng, Q., Baird, D., Peng, X., Wang, J., Ly, T., Guan, J. L., and Cerione, R. A. (2006) Cool-1 functions as an essential regulatory node for EGF receptor- and Src-mediated cell growth, *Nat Cell Biol 8*, 945-956.
15. Tu, S., Wu, W. J., Wang, J., and Cerione, R. A. (2003) Epidermal growth factor-dependent regulation of Cdc42 is mediated by the Src tyrosine kinase, *J Biol Chem 278*, 49293-49300.
16. Hart, M. J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W. D., Gilman, A. G., Sternweis, P. C., and Bollag, G. (1998) Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13, *Science 280*, 2112-2114.
17. Hoffman, G. R., and Cerione, R. A. (2000) Flipping the switch: the structural basis for signaling through the CRIB motif, *Cell 102*, 403-406.
18. Hoffman, G. R., and Cerione, R. A. (2002) Signaling to the Rho GTPases: networking with the DH domain, *FEBS Lett 513*, 85-91.
19. Nassar, N., Hoffman, G. R., Manor, D., Clardy, J. C., and Cerione, R. A. (1998) Structures of Cdc42 bound to the active and catalytically compromised forms of Cdc42GAP, *Nat Struct Biol 5*, 1047-1052.

20. Ahmadian, M. R., Stege, P., Scheffzek, K., and Wittinghofer, A. (1997) Confirmation of the arginine-finger hypothesis for the GAP-stimulated GTP-hydrolysis reaction of Ras, *Nat Struct Biol 4*, 686-689.

21. Hart, M. J., Maru, Y., Leonard, D., Witte, O. N., Evans, T., and Cerione, R. A. (1992) A GDP dissociation inhibitor that serves as a GTPase inhibitor for the Ras-like protein CDC42Hs, *Science 258*, 812-815.

22. Hoffman, G. R., Nassar, N., and Cerione, R. A. (2000) Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI, *Cell 100*, 345-356.

23. Johnson, J. L., Erickson, J. W., and Cerione, R. A. (2009) New insights into how The Rho-guanine nucleotide dissociation inhibitor regulates the interaction of CDC42 with membranes, *J Biol Chem*.

24. Gosser, Y. Q., Nomanbhoy, T. K., Aghazadeh, B., Manor, D., Combs, C., Cerione, R. A., and Rosen, M. K. (1997) C-terminal binding domain of Rho GDP-dissociation inhibitor directs N-terminal inhibitory peptide to GTPases, *Nature 387*, 814-819.

25. Srivastava, S. K., Wheelock, R. H., Aaronson, S. A., and Eva, A. (1986) Identification of the protein encoded by the human diffuse B-cell lymphoma (dbl) oncogene, *Proc Natl Acad Sci U S A 83*, 8868-8872.

26. Hart, M. J., Eva, A., Evans, T., Aaronson, S. A., and Cerione, R. A. (1991) Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the dbl oncogene product, *Nature 354*, 311-314.

27. Ron, D., Zannini, M., Lewis, M., Wickner, R. B., Hunt, L. T., Graziani, G., Tronick, S. R., Aaronson, S. A., and Eva, A. (1991) A region of proto-dbl essential for its transforming activity shows sequence similarity to a yeast cell

cycle gene, CDC24, and the human breakpoint cluster gene, bcr, *New Biol 3*, 372-379.

28. Hart, M. J., Eva, A., Zangrilli, D., Aaronson, S. A., Evans, T., Cerione, R. A., and Zheng, Y. (1994) Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product, *J Biol Chem 269*, 62-65.
29. Bi, F., Debreceni, B., Zhu, K., Salani, B., Eva, A., and Zheng, Y. (2001) Autoinhibition mechanism of proto-Dbl, *Mol Cell Biol 21*, 1463-1474.
30. Zheng, Y., Zangrilli, D., Cerione, R. A., and Eva, A. (1996) The pleckstrin homology domain mediates transformation by oncogenic dbl through specific intracellular targeting, *J Biol Chem 271*, 19017-19020.
31. Cerione, R. A., and Zheng, Y. (1996) The Dbl family of oncogenes, *Curr Opin Cell Biol 8*, 216-222.
32. Aghazadeh, B., Lowry, W. E., Huang, X. Y., and Rosen, M. K. (2000) Structural basis for relief of autoinhibition of the Dbl homology domain of proto-oncogene Vav by tyrosine phosphorylation, *Cell 102*, 625-633.
33. Feng, Q., Baird, D., and Cerione, R. A. (2004) Novel regulatory mechanisms for the Dbl family guanine nucleotide exchange factor Cool-2/alpha-Pix, *EMBO J 23*, 3492-3504.
34. Baird, D., Feng, Q., and Cerione, R. A. (2005) The Cool-2/alpha-Pix protein mediates a Cdc42-Rac signaling cascade, *Curr Biol 15*, 1-10.
35. Takai, S., Hasegawa, H., Kiyokawa, E., Yamada, K., Kurata, T., and Matsuda, M. (1996) Chromosomal mapping of the gene encoding DOCK180, a major Crk-binding protein, to 10q26.13-q26.3 by fluorescence in situ hybridization, *Genomics 35*, 403-404.

36. Hasegawa, H., Kiyokawa, E., Tanaka, S., Nagashima, K., Gotoh, N., Shibuya, M., Kurata, T., and Matsuda, M. (1996) DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane, *Mol Cell Biol 16*, 1770-1776.

37. Nolan, K. M., Barrett, K., Lu, Y., Hu, K. Q., Vincent, S., and Settleman, J. (1998) Myoblast city, the Drosophila homolog of DOCK180/CED-5, is required in a Rac signaling pathway utilized for multiple developmental processes, *Genes Dev 12*, 3337-3342.

38. Wu, Y. C., and Horvitz, H. R. (1998) C. elegans phagocytosis and cell-migration protein CED-5 is similar to human DOCK180, *Nature 392*, 501-504.

39. Cote, J. F., and Vuori, K. (2002) Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity, *J Cell Sci 115*, 4901-4913.

40. Brugnera, E., Haney, L., Grimsley, C., Lu, M., Walk, S. F., Tosello-Trampont, A. C., Macara, I. G., Madhani, H., Fink, G. R., and Ravichandran, K. S. (2002) Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex, *Nat Cell Biol 4*, 574-582.

41. Valles, A. M., Beuvin, M., and Boyer, B. (2004) Activation of Rac1 by paxillin-Crk-DOCK180 signaling complex is antagonized by Rap1 in migrating NBT-II cells, *J Biol Chem 279*, 44490-44496.

42. Fukui, Y. (2002) [A critical role of the CDM family molecule DOCK2 in lymphocyte migration], *Tanpakushitsu Kakusan Koso 47*, 2194-2199.

43. Namekata, K., Enokido, Y., Iwasawa, K., and Kimura, H. (2004) MOCA induces membrane spreading by activating Rac1, *J Biol Chem 279*, 14331-14337.

44. Yajnik, V., Paulding, C., Sordella, R., McClatchey, A. I., Saito, M., Wahrer, D. C., Reynolds, P., Bell, D. W., Lake, R., van den Heuvel, S., Settleman, J., and Haber, D. A. (2003) DOCK4, a GTPase activator, is disrupted during tumorigenesis, *Cell 112*, 673-684.

45. Hiramoto, K., Negishi, M., and Katoh, H. (2006) Dock4 is regulated by RhoG and promotes Rac-dependent cell migration, *Exp Cell Res 312*, 4205-4216.

46. Ueda, S., Fujimoto, S., Hiramoto, K., Negishi, M., and Katoh, H. (2008) Dock4 regulates dendritic development in hippocampal neurons, *J Neurosci Res 86*, 3052-3061.

47. Miyamoto, Y., Yamauchi, J., Sanbe, A., and Tanoue, A. (2007) Dock6, a Dock-C subfamily guanine nucleotide exchanger, has the dual specificity for Rac1 and Cdc42 and regulates neurite outgrowth, *Exp Cell Res 313*, 791-804.

48. Takahashi, K., Kohno, T., Ajima, R., Sasaki, H., Minna, J. D., Fujiwara, T., Tanaka, N., and Yokota, J. (2006) Homozygous deletion and reduced expression of the DOCK8 gene in human lung cancer, *Int J Oncol 28*, 321-328.

49. Yamauchi, J., Miyamoto, Y., Chan, J. R., and Tanoue, A. (2008) ErbB2 directly activates the exchange factor Dock7 to promote Schwann cell migration, *J Cell Biol 181*, 351-365.

50. Watabe-Uchida, M., John, K. A., Janas, J. A., Newey, S. E., and Van Aelst, L. (2006) The Rac activator DOCK7 regulates neuronal polarity through local phosphorylation of stathmin/Op18, *Neuron 51*, 727-739.

51. Pinheiro, E. M., and Gertler, F. B. (2006) Nervous Rac: DOCK7 regulation of axon formation, *Neuron 51*, 674-676.

52. Meller, N., Irani-Tehrani, M., Kiosses, W. B., Del Pozo, M. A., and Schwartz, M. A. (2002) Zizimin1, a novel Cdc42 activator, reveals a new GEF domain for Rho proteins, *Nat Cell Biol 4*, 639-647.

53. Nishikimi, A., Meller, N., Uekawa, N., Isobe, K., Schwartz, M. A., and Maruyama, M. (2005) Zizimin2: a novel, DOCK180-related Cdc42 guanine nucleotide exchange factor expressed predominantly in lymphocytes, *FEBS Lett 579*, 1039-1046.

54. Lin, Q., Yang, W., Baird, D., Feng, Q., and Cerione, R. A. (2006) Identification of a DOCK180-related guanine nucleotide exchange factor that is capable of mediating a positive feedback activation of Cdc42, *J Biol Chem 281*, 35253-35262.

55. Tosello-Trampont, A. C., Kinchen, J. M., Brugnera, E., Haney, L. B., Hengartner, M. O., and Ravichandran, K. S. (2007) Identification of two signaling submodules within the CrkII/ELMO/Dock180 pathway regulating engulfment of apoptotic cells, *Cell Death Differ 14*, 963-972.

56. van den Eijnde, S. M., Boshart, L., Baehrecke, E. H., De Zeeuw, C. I., Reutelingsperger, C. P., and Vermeij-Keers, C. (1998) Cell surface exposure of phosphatidylserine during apoptosis is phylogenetically conserved, *Apoptosis 3*, 9-16.

57. Vlassara, H., Valinsky, J., Brownlee, M., Cerami, C., Nishimoto, S., and Cerami, A. (1987) Advanced glycosylation endproducts on erythrocyte cell surface induce receptor-mediated phagocytosis by macrophages. A model for turnover of aging cells, *J Exp Med 166*, 539-549.

58. Savill, J., Hogg, N., Ren, Y., and Haslett, C. (1992) Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis, *J Clin Invest 90*, 1513-1522.

59. Park, D., Tosello-Trampont, A. C., Elliott, M. R., Lu, M., Haney, L. B., Ma, Z., Klibanov, A. L., Mandell, J. W., and Ravichandran, K. S. (2007) BAI1 is an

engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module, *Nature 450*, 430-434.

60. Kinchen, J. M., and Ravichandran, K. S. (2007) Journey to the grave: signaling events regulating removal of apoptotic cells, *J Cell Sci 120*, 2143-2149.

61. Reddien, P. W., and Horvitz, H. R. (2000) CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in Caenorhabditis elegans, *Nat Cell Biol 2*, 131-136.

62. Su, H. P., Nakada-Tsukui, K., Tosello-Trampont, A. C., Li, Y., Bu, G., Henson, P. M., and Ravichandran, K. S. (2002) Interaction of CED-6/GULP, an adapter protein involved in engulfment of apoptotic cells with CED-1 and CD91/low density lipoprotein receptor-related protein (LRP), *J Biol Chem 277*, 11772-11779.

63. Gardai, S. J., McPhillips, K. A., Frasch, S. C., Janssen, W. J., Starefeldt, A., Murphy-Ullrich, J. E., Bratton, D. L., Oldenborg, P. A., Michalak, M., and Henson, P. M. (2005) Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte, *Cell 123*, 321-334.

64. Gumienny, T. L., Brugnera, E., Tosello-Trampont, A. C., Kinchen, J. M., Haney, L. B., Nishiwaki, K., Walk, S. F., Nemergut, M. E., Macara, I. G., Francis, R., Schedl, T., Qin, Y., Van Aelst, L., Hengartner, M. O., and Ravichandran, K. S. (2001) CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration, *Cell 107*, 27-41.

65. Katoh, H., and Negishi, M. (2003) RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo, *Nature 424*, 461-464.

66. Lu, M., Kinchen, J. M., Rossman, K. L., Grimsley, C., Hall, M., Sondek, J., Hengartner, M. O., Yajnik, V., and Ravichandran, K. S. (2005) A Steric-inhibition model for regulation of nucleotide exchange via the Dock180 family of GEFs, *Curr Biol 15*, 371-377.

67. Grimsley, C. M., Kinchen, J. M., Tosello-Trampont, A. C., Brugnera, E., Haney, L. B., Lu, M., Chen, Q., Klingele, D., Hengartner, M. O., and Ravichandran, K. S. (2004) Dock180 and ELMO1 proteins cooperate to promote evolutionarily conserved Rac-dependent cell migration, *J Biol Chem 279*, 6087-6097.

68. Yin, J., Haney, L., Walk, S., Zhou, S., Ravichandran, K. S., and Wang, W. (2004) Nuclear localization of the DOCK180/ELMO complex, *Arch Biochem Biophys 429*, 23-29.

69. Makino, Y., Tsuda, M., Ichihara, S., Watanabe, T., Sakai, M., Sawa, H., Nagashima, K., Hatakeyama, S., and Tanaka, S. (2006) Elmo1 inhibits ubiquitylation of Dock180, *J Cell Sci 119*, 923-932.

70. deBakker, C. D., Haney, L. B., Kinchen, J. M., Grimsley, C., Lu, M., Klingele, D., Hsu, P. K., Chou, B. K., Cheng, L. C., Blangy, A., Sondek, J., Hengartner, M. O., Wu, Y. C., and Ravichandran, K. S. (2004) Phagocytosis of apoptotic cells is regulated by a UNC-73/TRIO-MIG-2/RhoG signaling module and armadillo repeats of CED-12/ELMO, *Curr Biol 14*, 2208-2216.

71. Akakura, S., Singh, S., Spataro, M., Akakura, R., Kim, J. I., Albert, M. L., and Birge, R. B. (2004) The opsonin MFG-E8 is a ligand for the alphavbeta5 integrin and triggers DOCK180-dependent Rac1 activation for the phagocytosis of apoptotic cells, *Exp Cell Res 292*, 403-416.

CHATPER 2

A NEW FUNCTIONAL LIMIT DOMAIN OF DOCK180

2.1 Introduction

Dock180 was originally identified as an unconventional Rac-GEF protein because it lacked the tandem DH-PH domain, which is a hallmark of all Dbl-GEF family members. A family of GEFs for Rac and Cdc42 was subsequently discovered for which the founding member is Dock180. This family of GEFs includes eleven members in mammalian cells and a number of orthologues in yeast, worms, flies and other species. These proteins share two conserved domains, designated as DHR-1 (Dock180 homology region-1) and DHR-2 (*2*). The DHR-2 domain comprises approximately 500 amino acids and responsible for the GEF activity of Dock180. As discussed in the previous chapter, Dock180 family can be further divided into four sub-families. As shown in Figure 2.1, the sequence identity for the DHR-2 domain among members of a given subfamily is high (i.e., the identity of DHR-2 between Dock180 and Dock2 is more than 70%), whereas the identity between members of different sub-families is quite low (i.e. the identity of DHR-2 between Dock180 (Dock A subfamily) and Dock7 (Dock C subfamily) is less than 15%). However, based on secondary structural predictions, the secondary structure of the DHR-2 domain of Dock180 and Dock7 is similar as shown in Figure 2.2, suggesting that they share a common tertiary structure and activation mechanism for their GEF activity.

Several groups have already successfully expressed the DHR-2 domain of Dock180 and other family members *in vitro* and *in vivo*. It has been shown that the

Figure 2.1 Primary sequence alignments of the DHR-2 domains from the Dock180 superfamily. The amino acids used from the respective sequences are as follows: Dock7 (1257-1894), Dock2 (1092-1670), and Dock180 (1088-1672).

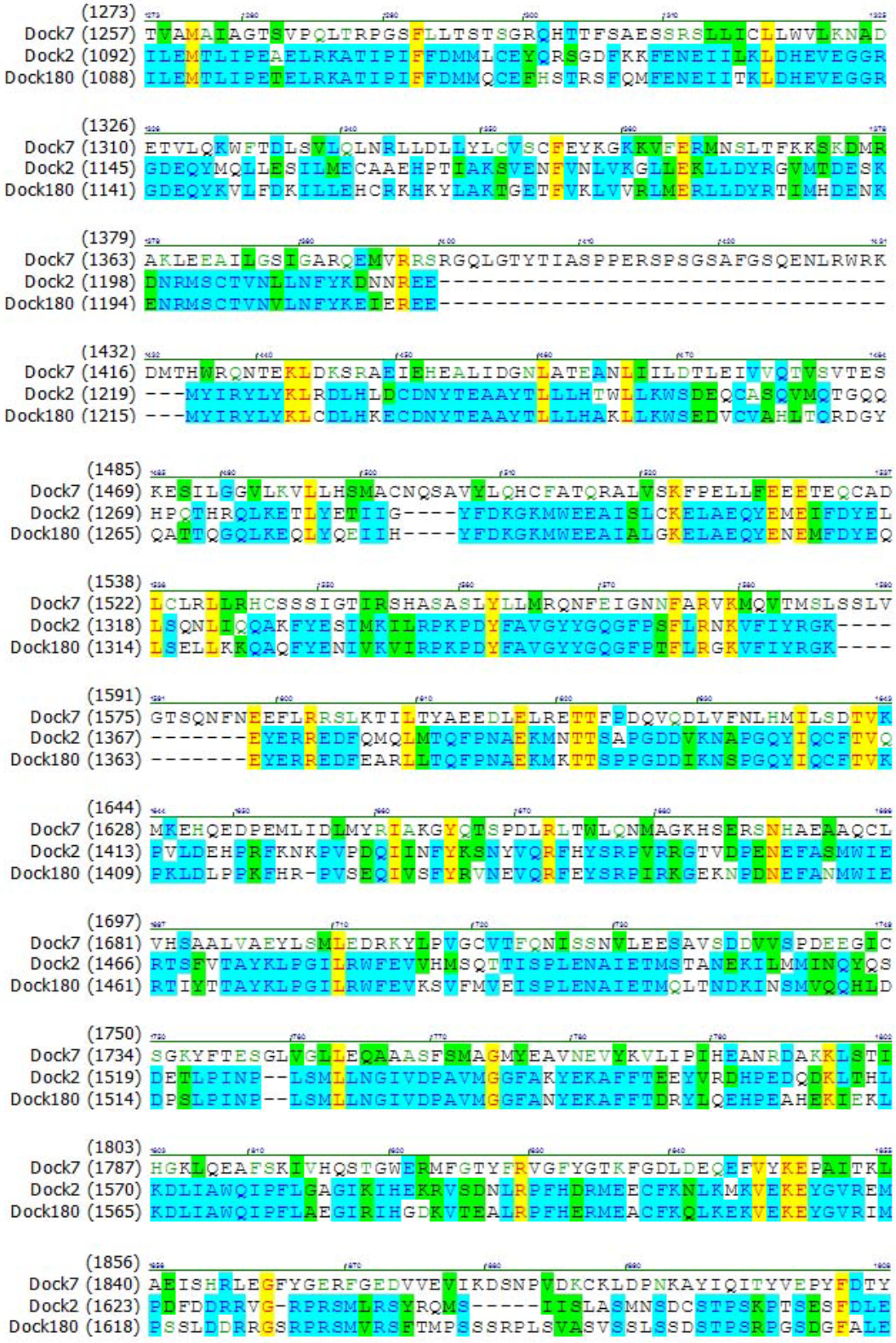

(1273)
Dock7 (1257) TVAMAIAGTSVPQLTRPGSFLLTSTSGRQHTTFSAESSRSLLICLLWVLKNAD
Dock2 (1092) ILEMTLIPEAELRKATIPIFFDMMLCEYQRSGDFKKFENEIILKLDHEVEGGR
Dock180 (1088) ILEMTLIPETELRKATIPIFFDMMQCEFHSTRSFQMFENEIITKLDHEVEGGR
(1326)
Dock7 (1310) ETVLQKWFTDLSVLQLNRLLDLLYLCVSCFEYKGKKVFERMNSLTFKKSKDMR
Dock2 (1145) GDEQYMQLLESILMECAAEHPTIAKSVENFVNLVKGLLEKLLDYRGVMTDESK
Dock180 (1141) GDEQYKVLFDKILLEHCRKHKYLAKTGETFVKLVVRLMERLLDYRTIMHDENK
(1379)
Dock7 (1363) AKLEEAILGSIGARQEMVRRSRGQLGTYTIASPPERSPSGSAFGSQENLRWRK
Dock2 (1198) DNRMSCTVNLLNFYKDNNREE----------
Dock180 (1194) ENRMSCTVNVLNFYKEIEREE----------
(1432)
Dock7 (1416) DMTHWRQNTEKLDKSRAEIEHEALIDGNLATEANLIILDTLEIVVQTVSVTES
Dock2 (1219) ---MYIRYLYKLRDLHLDCDNYTEAAYTLLLHTWLLKWSDEQCASQVMQTGQQ
Dock180 (1215) ---MYIRYLYKLCDLHKECDNYTEAAYTLLLHAKLLKWSEDVCVAHLTQRDGY
(1485)
Dock7 (1469) KESILGGVLKVLLHSMACNQSAVYLQHCFATQRALVSKFPELLFEETEQCAD
Dock2 (1269) HPQTHRQLKETLYETIIG----YFDKGKMWEEAISLCKELAEQYEMEIFDYEL
Dock180 (1265) QATTQGQLKEQLYQEIIH----YFDKGKMWEEAIALGKELAEQYENEMFDYEQ
(1538)
Dock7 (1522) LCLRLLRHCSSSIGTIRSHASASLYLLMRQNFEIGNNFARVKMQVTMSLSSLV
Dock2 (1318) LSQNLIQQAKFYESIMKILRPKPDYFAVGYYGQGFPSFLRNKVFIYRGK----
Dock180 (1314) LSELLKKQAQFYENIVKVIRPKPDYFAVGYYGQGFPTFLRGKVFIYRGK----
(1591)
Dock7 (1575) GTSQNFNEEFLRRSLKTILTYAEEDLELRETTFPDQVQDLVFNLHMILSDTVK
Dock2 (1367) -------EYERREDFQMQLMTQFPNAEKMNTTSAPGDDVKNAPGQYIQCFTVQ
Dock180 (1363) -------EYERREDFEARLLTQFPNAEKMKTTSPPGDDIKNSPGQYIQCFTVK
(1644)
Dock7 (1628) MKEHQEDPEMLIDLMYRIAKGYQTSPDLRLTWLQNMAGKHSERSNHAEAAQCL
Dock2 (1413) PVLDEHPRFKNKPVPDQIINFYKSNYVQRFHYSRPVRRGTVDPENEFASMWIE
Dock180 (1409) PKLDLPPKFHR-PVSEQIVSFYRVNEVQRFEYSRPIRKGEKNPDNEFANMWIE
(1697)
Dock7 (1681) VHSAALVAEYLSMLEDRKYLPVGCVTFQNISSNVLEESAVSDDVVSPDEEGIC
Dock2 (1466) RTSFVTAYKLPGILRWFEVVHMSQTTISPLENAIETMSTANEKILMMINQYQS
Dock180 (1461) RTIYTTAYKLPGILRWFEVKSVFMVEISPLENAIETMQLTNDKINSMVQQHLD
(1750)
Dock7 (1734) SGKYFTESGLVGLLEQAAASFSMAGMYEAVNEVYKVLIPIHEANRDAKKLSTI
Dock2 (1519) DETLPINP--LSMLLNGIVDPAVMGGFAKYEKAFFTEEYVRDHPEDQDKLTHL
Dock180 (1514) DPSLPINP--LSMLLNGIVDPAVMGGFANYEKAFFTDRYLQEHPEAHEKIEKL
(1803)
Dock7 (1787) HGKLQEAFSKIVHQSTGWEPMFGTYFRVGFYGTKFGDLDEQEFVYKEPAITKL
Dock2 (1570) KDLIAWQIPFLGAGIKIHEKRVSDNLRPFHDRMEECFKNLKMKVEKEYGVREM
Dock180 (1565) KDLIAWQIPFLAEGIRIHGDKVTEALRPFHERMEACFKQLKEKVEKEYGVRIM
(1856)
Dock7 (1840) AEISHRLEGFYGERFGEDVVEVIKDSNPVDKCKLDPNKAYIQITYVEPYFDTY
Dock2 (1623) PDFDDRRVG-RPRSMLRSYRQMS-----IISLASMNSDCSTPSKPTSESFDLE
Dock180 (1618) PSSLDDRRGSRPRSMVRSFTMPSSSRPLSVASVSSLSSDSTPSRPGSLGFALE

Figure 2.2 Secondary structural predictions for the DHR-2 domain of Dock180 and Dock7 using J-pred.

Dock7 DHR2

```
MRQNFEIGNNFARVKMQVTMSLSSLVGTSQNFNEEFLRRSLKTILTYAEEDLELRETTFPDQ
VQDLVFNLHMILSDTVKMKEHQEDPEMLIDLMYRIAKGYQTSPDLRLTWLQNMAGKHSERSN
HAEAAQCLVHSAALVAEYLSMLEDRKYLPVGCVTFQNISSNVLEESAVSDDVVSPDEEGICS
GKYFTESGLVGLLEQAAASFSMAGMYEAVNEVYKVLIPIHEANRDAKKLSTIHGKLQEAFSK
IVHQSTGWERMFGTYFRVGFYGTKFGDLDEQEFVYKEPAITKLAEISHRLEGFYGERFGEDV
VEVIKDSNPVDKCKLDPNKAYIQITYVEPYFDTYEMKDRITYFDKNYNLRRFMYCTPFTLDG
RAHGELHEQFKRKTILTTSHAFPYIKTRVNVTHKEEIILTPIEVAIEDMQKKTQELAFATHQ
DPADPKMLQMVLQGSVGTTVNQGPLEVAQVFLSEIPSDPKLFRHHNKLRLCFKDFTKRCEDA
LRKNKSLIGPDQKEYQRELERNYHRLKEALQPLINRKIPQLYKAVLPVTCHRDSFSRMSLRK
MDL
-----HH-----HHHHHHHHHHHHHHH-------HHHHHHHHHHHHHHHH---------
HHHHHHHHHHHH HHHHHHHHHHH----HHHHHHHHHHHHHH----
HHHHHHHHHHHHHHHHH----HHHHHHHHHHHHHHHHH HH--------------------
------------------------HHHHHHHHHHHHHHHHH---HH
HHHHHHHHHHHHHHH---HHHHHHHHHHHHHHHHHHHH-----------EEEEEEE------
----EEEEE ------HHHHHHHHHHH-------EEEE----------------
EEEEEEEEEE-------------EE ---EEEEEEEEEEE----------EEEEEEEEEEE-
---------EEEEEEEEEEE—HHHHHHHHHHHHHH HHHHHH-------HHHHHHHHH----
----HHHHHHHHH---------HHHHHHHHHHHHHHHHHHHHHHH HHHH---
HHHHHHHHHHHHHHHHHHHHHHHHH--------------------------E---
```

Dock180 DHR2

```
MQCEFHSTRSFQMFENEIITKLDHEVEGGRGDEQYKVLFDKILLEHCRKHKYLAKTGETFVK
LVVRLMERLLDYRTIMHDENKENRMSCTVNVLNFYKEIEREEMYIRYLYKLCDLHKECDNYT
EAAYTLLLHAKLLKWSEDVCVAHLTQRDGYQATTQGQLKEQLYQEIIHYFDKGKMWEEAIAL
GKELAEQYENEMFDYEQLSELLKKQAQFYENIVKVIRPKPDYFAVGYYGQGFPTFLRGKVFI
YRGKEYERREDFEARLLTQFPNAEKMKTTSPPGDDIKNSPGQYIQCFTVKPKLDLPPKFHRP
VSEQIVSFYRVNEVQRFEYSRPIRKGEKNPDNEFANMWIERTIYTTAYKLPGILRWFEVKSV
FMVEISPLENAIETMQLTNDKINSMVQQHLDDPSLPINPLSMLLNGIVDPAVMGGFANYEKA
FFTDRYLQEHPEAHEKIEKLKDLIAWQIPFLAEGIRIHGDKVTEALRPFHERMEACFKQLKE
KVEKEYGVRIMPSSLDDRRGSRPRSMVRSFTMPSSSRPLSVASVSSLSSD
----HE----HHHHHHHHHHHHHHH-------HHHHHHHHHHHHHHH---------
HHHHHHHHHHHHHHH HHH--------HHHHHHHHHHHHHHHHH----
HHHHHHHHHHHHHHHHH----HHHHHHHHHHHHHH---------------------
HHHHHHHHHHHHHHHHH---HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
HHHHHHH-------EEEEEEE-----------EEEEE------HHHHHHHHHH-----EE-
---------------EEEEEEEEEEE---------------EEEE----EEEEEEEE----
----------EEEEEEEE E-----------EEEEEEEEEE-
HHHHHHHHHHHHHHHHHHHHHHH----------HHHHHHH----------HHHHHHHH-----
-------HHHHHHHHHHHHHHHHHHHHHHHHHHHHH—HHHHHHHHHHHHHHHHHHHH
HHHH----------------------------------------
```

DHR-2 domain binds to and activates Rac *in vitro* and is also necessary and sufficient for the Dock180-mediated activation of Rac *in vivo*, while other domains (the SH-3, DHR-1, and proline-rich domains) of Dock180 do not appear to directly contribute to Rac activation. Experiments performed *in vitro* have also shown that the DHR-2 domain exhibits significantly higher GEF activity than the full-length Dock180 which is subject to auto-inhibition (*3*).

Although the DHR-2 domain of Dock180 has been expressed in *E.coli* by several groups, it has been difficult to isolate the DHR-2 domains and typically only very low yields of protein have been obtained (100 μg level from 1 L culture). Efforts have been made to identify possible sub-domains of DHR-2 by secondary structure analysis. It was claimed that DHR-2 might contain a 'DH' domain (i.e. within amino acids 1111 to 1335) and a 'PH' domain (amino acids1395-1515) (*4*). However, none of these sub-domains have shown GEF activity toward Rac. Based on these results, it has been concluded that the entire DHR-2 domain (including amino acids 1111 to 1657) is necessary for Rac activation.

However, when we began to examine and characterize the DHR-2 domain, we obtained some results that were not consistent with previous published work. In particular, we identified a more limit sub-domain of DHR-2 which is well expressed in *E.coli*. Either *in vitro* or *in vivo*, this sub-domain can fully replace the GEF activity of Dock180. And it is much stable than the full-length DHR-2c and shows specific GEF activity towards Rac. Our fluorescence data demonstrated that this sub-domain showed relatively high GEF activity comparing to classical Dbl-GEF proteins.

2.2 Methods

Plasmids. The Dock180 plasmid was a gift from Michiyuki Matsyda. To obtain the clone of the original DHR-2 domain (amino acids 1178-1657), the polymerase chain reaction (PCR) was performed using the Dock180 plasmid as template DNA and primers 5' –GCGGATCCATGGAAAGGC TTTTGGAT -3' and 5'-CGGAATTCTCACGATGAG AGGGAAGAGA-3'. The PCR product was cloned into the pET28a plasmid using BamH I and EcoR I restriction sites. The DHR-2n sub-domain (1178-1334) and the DHR-2c sub-domain (1135-1657) constructs were generated by PCR using DHR-2 as the template and cloned into both pET28a and pKH-3 plasmids.

Protein Expression and Purification. Single colonies of *E.coli* BL21 (DE3) containing target plasmids were inoculated in 10 ml of LB media with 50 µg/ml kanamysin or carbenicillin (RPI) and cultured overnight at 37°C. These small cultures were subsequently used to inoculate 1 L LB media with 50 µg/ml kanamysin/ carbenicillin in a shaking incubator at 37°C. The large-scale cultures were incubated to a density of OD_{600}=0.6 and induced by IPTG (RPI) (final concentration = 200 µM) at room temperature overnight. Bacteria were harvested by centrifugation at 5000 rpm for 10 minutes and the pellet was re-suspended in lysis buffer (20 mM Tris-HCl PH 8.0, 5 mM $MgCl_2$, 500 mM NaCl) with 10 µg/ml leupeptin and 10 µg/ml aprotinin.

For DHR-2, DHR-2c and DHR-2n, the suspensions were sonicated and the lysates were cleared by centrifugation at 20000 rpm for 30 minutes. The supernatants were collected and incubated with nickel chelate beads (Amersham) for 30 minutes on ice. The beads were washed with lysis buffer containing 40 mM imidazole until there was no detectable protein in the wash buffer. The proteins were eluted with lysis

buffer containing 200 mM imidazole, concentrated to 200 µM, and stored at -80°C for further use.

Rac and Cdc42 were expressed using similar procedures as described above and the supernatants were collected and applied to a glutathione-sepharose column. Unbound proteins were washed off by lysis buffer and target proteins were eluted using the same buffer with 10 mM glutathione. The eluted proteins were applied to a P-10 desalting column to remove glutathione and concentrated to 300 µM. Mant-GDP-Rac and mant-GDP-Cdc42 were prepared by mixing Rac with a 10-fold excess of mant-GDP in lysis buffer with 10 mM EDTA for 10 minutes. Excess $MgCl_2$ was added to saturate EDTA. The mixture was applied to a P-10 desalting column equilibrated in lysis buffer in order to remove unbound mant-GDP.

In order to obtain highly purified protein for crystallization, DHR-2c, bound to nickel beads, was incubated with thrombin (Haemotologic Tech) (1 unit for 3 mg target protein) to digest the His-tag overnight at 4°C. The flow-through was collected, concentrated and applied to a HiTrap Q HP column using AKTA FPLC (GE). DHR-2c does not bind to the Q column and so the flow-through was collected and concentrated to a volume of 5 ml. The concentrated protein was then applied to a G-75 gel-filtration column which was pre-equilibrated with crystallization buffer (Tris-HCl 20 mM pH 8.0, 100 mM NaCl). The fractions containing DHR-2c were examined by SDS-PAGE gel. The highly purified DHR-2c was concentrated to 10 mg/ml for crystallization screens.

High quality His-Tag Rac was purified in a similar manner except that $MgCl_2$ (5 mM) was present in solution throughout the purification. Purified Rac and DHR-2c were mixed at the molar ration 2:1 in crystallization buffer with 10 mM EDTA for 30 minutes at 4°C. The mixture of Rac and DHR-2c was then applied to a G-200 gel-

filtration column. The protein complex was identified by SDS-PAGE and concentrated to 10 mg/ml for crystallization screens.

In vitro GEF Assay. All fluorescence measurements were performed using a Varian Ecilpse Fluorescence Spectrophotometer. Samples were stirred continuously and thermostated at 25°C in HMA buffer (20 mM HEPES pH 8.0, 5 mM $MgCl_2$, 1 mM NaN_3). *In vitro* GEF assays using mant-GDP as a probe to monitor fluorescent changes accompanying nucleotide exchange were performed on recombinant GTPases. Mant-GDP was added to HMA buffer to a final concentration of 1 µM. Different concentration of Rho GTPases (Rac, Cdc42) and their mutants were added together with various concentrations of GEF proteins (DHR-2, DHR-2c). The Mant-GDP fluorescence changes were monitored using an excitation wavelength of 340 nm and an emission wavelength of 440 nm at 25°C. Each measurement was repeated at least three times. When measuring the turn-over rate of the GEF proteins, Rac were preloaded with mant-GDP. The decrease of fluorescence was detected when different concentrations of Rac were mixed with the GEF proteins and excess GDP. The calibration of the decrease in fluorescence yielded the *k*cat value for the GEF proteins.

GST-Rac pull-down assay. To check the binding of DHR-2 and DHR-2c with Rac, GST-Rac (0.3 nmol) were preloaded on 15 µl of Glutathione Sepharose beads. An equivalent amount of DHR-2c was added to the beads with 5 mM EDTA. The negative control tube only contained beads and DHR-2c (no GST-Rac). The tubes containing these mixtures were rotated at 4°C for half hour and then centrifuged at 12000 rpm for 1 min. The supernatant was discarded. The beads were washed with buffer (three times) and loaded onto an SDS-PAGE gel. Bound DHR-2c was detected by Coomassie blue staining.

To check the nucleotide-binding preference, glutathione Sepharose beads preloaded with GST-Rac were mixed with DHR-2c and excess GDP/GTP• S in EDTA buffer. Excess $MgCl_2$ was added to the solution after 15 minutes and incubated for an additional 15 minutes. The beads were washed and binding was detected as described above.

Indirect Immunofluorescence. The cells were transfect by with plasmids of interest and then plated on acid coverslips overnight. The cells were fixed on the coverslip in 4% paraformaldehyde for 20 minutes at room temperature and then rinsed with PBS three times. Triton X-100 (0.1%) was added to the permeabilized cells. The cells were rinsed with PBS an additional three times, and then incubated with primary and secondary antibodies in blocking buffer (10% serum in PBS). The cells were again washed with PBS (3X) after each incubation.

PBD Assays. Cells transfected with plasmids of interest were harvested with MBL buffer (Magnesium-containing lysis buffer) containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 10 µg/ml leupeptin and 10 µg/ml aprotinin. Cell extracts were cleared by centrifugation, and the supernatants were used to assess the total amount of Rac. The remaining extract was combined with 2 volumes of lysis buffer and the bacterially produced Rac/Cdc42- binding domain of Pak (i.e. designated as GST-PBD (Upstate)) coupled to glutathione beads and incubated for 30 minutes at 4 °C. The beads were washed (three times) with lysis buffer and eluted in 5× SDS-Loading sample buffer. Aliquots of both total cell extracts and the eluents from the PBD beads were blocked by anti-Myc.

2.3 Results

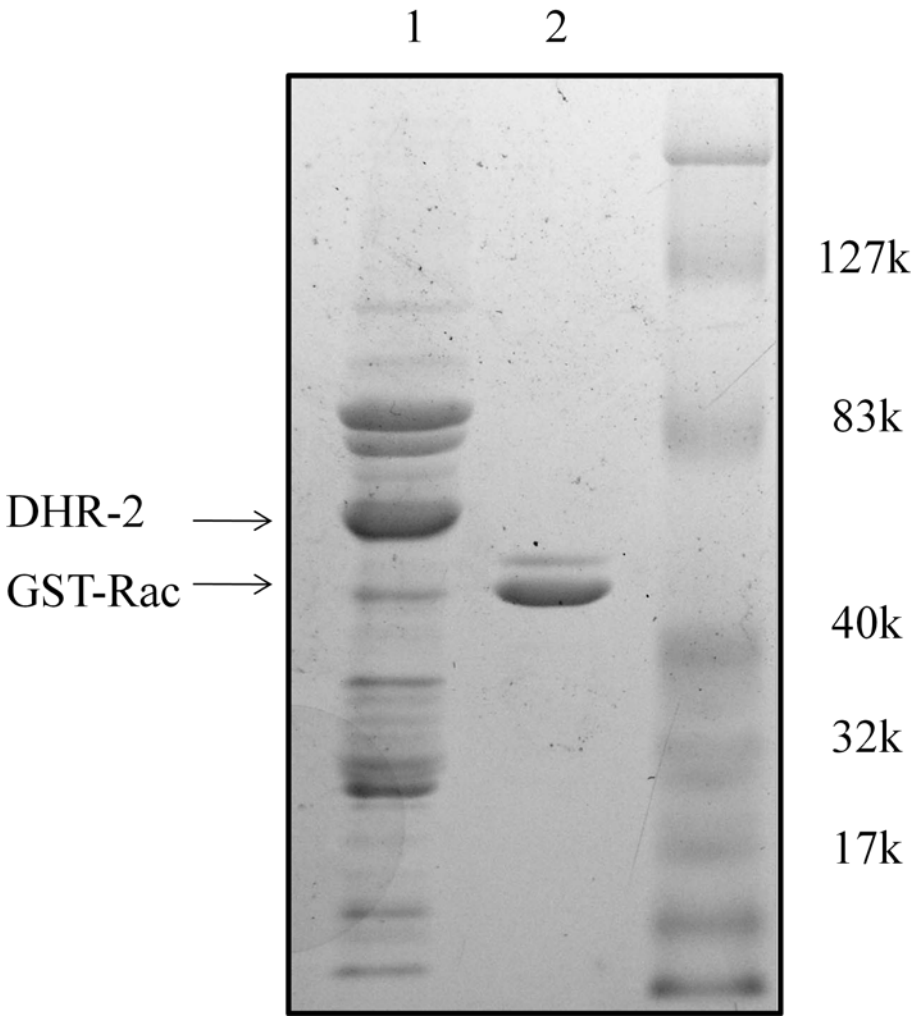
2.3.1 The DHR-2 domain can activate Rac *in vitro*

The DHR-2 domain of Dock180 was first cloned and characterized in 2002 (*2*). The original DHR-2 domain includes amino acids 1111 to 1657 and is conserved in all Dock180 family members. This DHR-2 construct was widely used by all groups who were working on the characterization of Dock180 function. It was claimed that the DHR-2 domain can activate Rac both *in vitro* and *in vivo(4)*. However, the expression level of recombinant DHR-2 in *E.coli* is very low.

While some of the initial reports on Dock180 appeared, the DHR-2 domain of Dock180 was cloned and expressed in bacteria by our laboratory. This DHR-2 construct is shorter at N-terminus by 70 amino acids compared to the original DHR-2c construct described above (*2*), with an apparent molecular weight of approximately 56 kDa. We successfully expressed this domain in BL21 DE3 cells using both GST-4T-1 and pET28a plasmids. Normally, GST-tagged proteins are more soluble than His-tagged proteins because the GST-tag can help facilitate proper protein folding and prevent the protein of interest from forming inclusion bodies. However, for the DHR-2 domain of Dock180, His-tagged DHR-2 behaviors better in solution than the GST-tagged protein. Thus, the expression levels of our His-DHR-2 construct (~6mg from a 2 L culture) are much higher than those for the longer form of DHR-2 that was often used in the past (~ 100 µg from a 2 L culture) (Figure 2.3). Therefore, it appears that the truncation of the first 70 amino acid enhances the protein expression and increases the solubility of the DHR-2 domain.

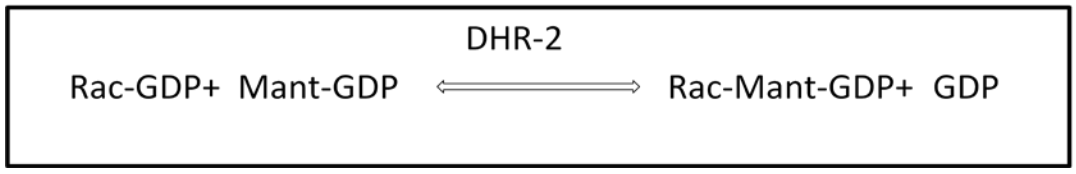
To test the activity of the truncated His-DHR-2 construct, we took advantage of a fluorescent read-out using mant-GDP to assay nucleotide exchange on Rac as

Figure 2.3 DHR-2 can be expressed in the pET28a plasmid in BL21 DE3 cells. Lane1 shows the purified DHR-2 protein after nickel-affinity chromatography. The size of DHR-2 is approximately 60 kDa compared to the GST-Rac protein (Mr ~ 52 kDa) shown in lane 2.


1
2
127k
83k
DHR-2
GST-Rac
40k
32k
17k

catalyzed by the DHR-2 domain *in vitro*. Mant-GDP is an analogue of GDP in which the ribose 2'-hydroxy or the 3'hydroxy group has been esterified by the fluorescent methylisatoic acid. Mant has a maximum excitation wavelength at 355 nm and a maximum emission wavelength at 448 nm. We used an excitation wavelength of 340 nm and an emission wavelength of 440 nm in all of our experiments. Because mant-GDP shows much higher fluorescence when bound to GTPases like Rac, compared to when it is free in solution, the fluorescence read-out provides a highly sensitive assay for nucleotide exchange (i.e. the exchange of GDP for mant-nucleotide).

The exchange is initiated by mixing mant-GDP with Rac (or Cdc42 or their mutants). Because of the low intrinsic rate of GDP dissociation from Rac, mant-

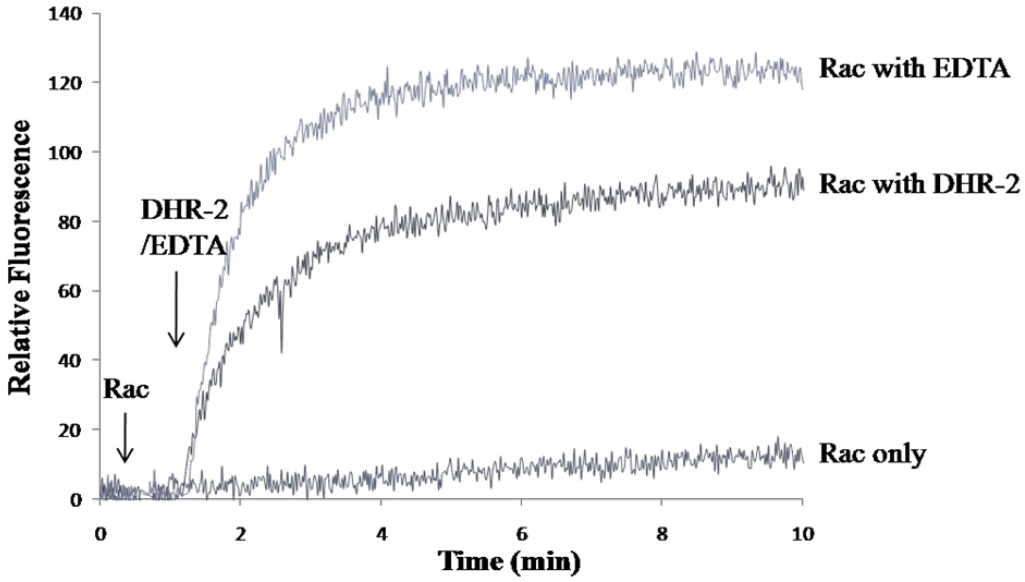


Equation 2.1

fluorescence is not significantly increased in the absence of GEF proteins. After adding a Rac-GEF, Rac will form a complex with the GEF, resulting in a weakening of the affinity for GDP such that it dissociates from Rac.

As depicted in Equation 2.1 above, mant-GDP can replace the dissociated GDP on Rac, giving rise to a fluorescence increase. Figure 2.4 shows that there was no significant increase in mant-fluorescence when GST-Rac was added to a solution containing mant-GDP. After DHR-2 was added, the mant-fluorescence increased rapidly. This *in vitro* fluorescence GEF assay confirmed that the recombinant DHR-2 domain is active and responsible for the GEF activity of the Dock180 protein.

Figure 2.4 DHR-2 activates Rac *in vitro*. 100 nM DHR-2 was mixed with 600 nM Rac1 in HMA buffer with 1 µM Mant-GDP. Nucleotide exchange was detected by monitoring increase in mant-fluorescence. The addition of EDTA served as a positive control for the GEF assay. It is well established that, EDTA weakens the affinity of small GTPases for GDP and therefore the presence of excess GTP, EDTA stimulates GDP-GTP exchange.


140
120
100
80
60
40
20
0
Relative Fluorescence
0
2
4
6
8
10
Time (min)
Rac with EDTA
Rac with DHR-2
Rac only
DHR-2
/EDTA
Rac

We also confirmed that DHR-2 showed the characteristics of a GEF by using an *in vitro* pull-down assay. This assay takes advantage of the limit Cdc42/Rac-binding domain of the PAK protein (called the PBD) which is fused to GST. GST-beads pre-loaded with GST-PBD protein can bind and pull-down GTP-bound Rac, but not GDP-bound Rac. Using this assay, we found that the DHR-2 domain can promote GDP-GTP exchange on Rac. Specifically, Figure 2.5 showed that much more GTP was loaded onto Rac (lane 3) in the presence of DHR-2c compared to the amount of Rac-GTP formed in the absence of DHR-2 (lane 2).

Dock180 and its functional domain DHR-2 were reported to show Rac-specific activity. Using the mant-fluorescence assay for nucleotide exchange, we confirmed that the DHR-2 domain was unable to activate Cdc42 under conditions where it clearly activated Rac (Figure 2.6).

As a GEF protein, the DHR-2 domain preferentially binds to nucleotide-free Rac. The GST-Rac pull-down assay showed that the binding of DHR-2 to nucleotide-free Rac (Figure 2.7 lane 2) is much stronger than to either GDP- or GTP-bound forms of Rac (lanes 3 and 4).

Our preliminary data suggested that the DHR-2 domain can be well expressed in *E.coli*. However, the DHR-2 domain was still only partially purified following nickel-affinity chromatography (see Figure 2.3). A significant effort was made to improve the purification of DHR-2. However, when we applied the protein (following affinity column) to a G200 gel-filtration column, equilibrated in 20 mM Tris-HCl pH8.0 and 100 mM NaCl, the majority of DHR-2 was found in the void volume, indicating that it aggregated during purification. We found that the aggregation was caused by the instability of the DHR-2 domain in low salt solution. After we gradually increased the concentration of NaCl in the buffer, a portion of DHR-2 was eluted

Figure 2.5 *In vitro* PBD assay showing that recombinant DHR-2 promotes the GTP-bound form of Rac. 1 µg His-Rac and 200 ng DHR-2 were incubated with excess GTP in HMA buffer with 25 µl PBD-linked beads for 30 minutes. The beads were washed several times and the Rac-GTP bound to the PBD beads was detected by western blotting with an anti-His antibody. Lane 3 showed that GTP loading on Rac was much greater in the presence of DHR-2 compared to when DHR-2 was not present (lane 2).

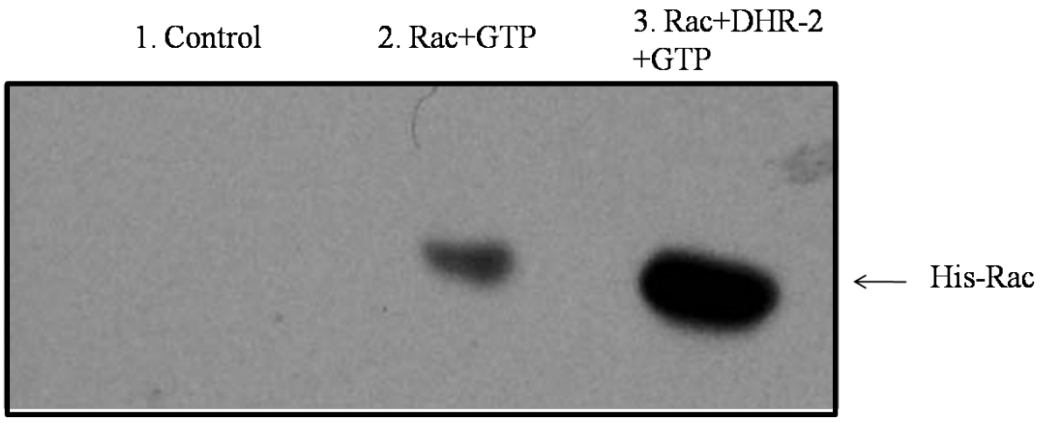

1. Control
2. Rac+GTP
3. Rac+DHR-2
+GTP
His-Rac

Figure 2.6 DHR-2 specifically activates Rac. Equal concentrations of Rac and Cdc42 were mixed with DHR-2 and mant-GDP. Rac showed a significant fluorescent change in the presence of DHR-2, while Cdc42 did not show detectable mant-GDP binding due to its exchange for GDP on Rac. EDTA servers as a positive control as it stimulates GDP-GTP exchange on small GTPases.

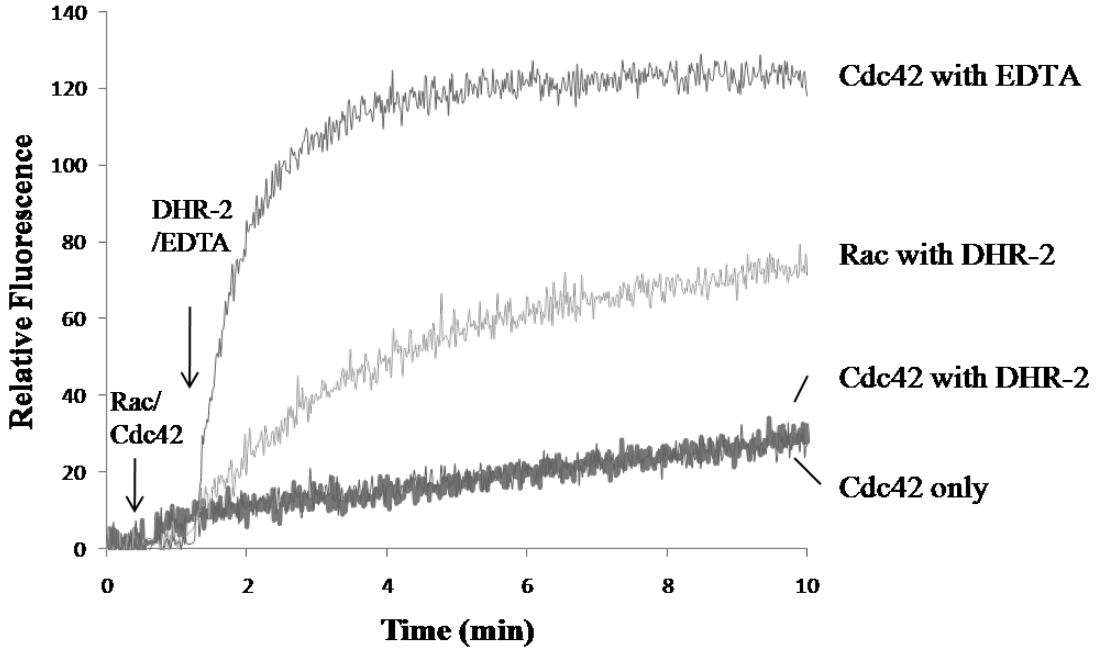
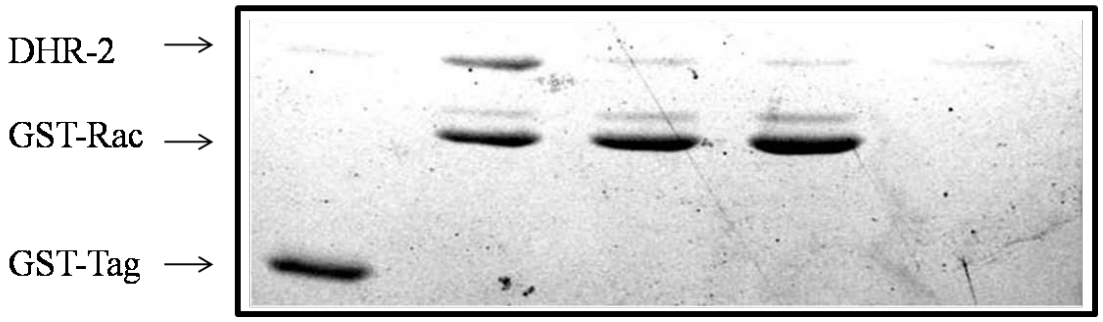

140
120
100
80
60
40
20
0
Relative Fluorescence
0
2
4
6
8
10
Time (min)
DHR-2
/EDTA
Rac/
Cdc42
Cdc42 with EDTA
Rac with DHR-2
Cdc42 with DHR-2
Cdc42 only

Figure 2.7 DHR-2 selectively bound to the nucleotide-free (NF) form of Rac. GST-Rac was immobilized on GST-beads and preloaded with GDP or GTP, or incubated in the absence of nucleotide. Equivalent mounts of DHR-2 were added to each incubation. The binding of DHR-2 to Rac was detected by Coomassie blue staining. The DHR-2 domain binds more effectively to nucleotide-free Rac (lane 2) compared to either GDP or GTP-bound Rac (lanes 3 and 4).

DHR-2	+	+	+	+	+
NF GST-Rac	--	+	+	+	--
GDP	--	--	+	--	--
GTP	--	--	--	+	--
GST-Tag	+	--	--	--	--



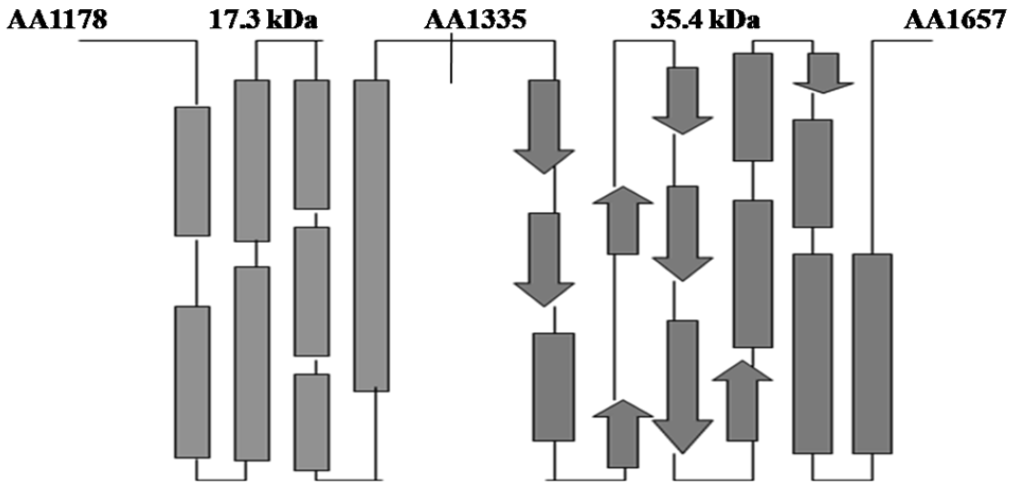
through gel-filtration as a monomer although the majority of the protein was still aggregated. The amount of monomeric DHR-2 was not sufficient for further purification and characterization. We also tried to form a complex between DHR-2 and Rac to see whether Rac can help stabilize DHR-2. The results showed that the complex had a similar tendency to aggregate. Because of its instability in low salt solution and the problem with aggregation, it has not been possible to perform a rigorous structure-function characterization of this GEF domain. To overcome this technical difficulty, we attempted to look for a shorter (limit) functional domain of Dock180.

2.3.2 A new limit DHR-2 domain was discovered

As we have described above, although the DHR-2 domain can be well expressed in bacteria and activate Rac *in vitro*, it aggregates in low salt solution. We suspected that a hydrophobic region on DHR-2, which was normally involved in an interaction with another region of full-length Dock180, became exposed in DHR-2 and underwent aggregation. If the region on DHR-2 responsible for aggregation does not influence its GEF activity, then truncation of this region may help improve the stability of the resultant DHR-2 domain construct.

Secondary structural analysis using J-pred revealed that there are two sub-domains in DHR-2 (Figure 2.8). The first sub-domain consists of amino acids 1178-1335 at the N-terminal of DHR-2 (from here on designated DHR-2n). This region is comprised entirely of • -helices. The second sub-domain begins at amino acid 1336 and extends to the C-terminus of DHR-2 (designated DHR-2c) and is comprised of • -

Figure 2.8 Secondary structural predictions of the DHR-2 domain of Dock180 using J-pred revealed two different sub-domains (residues1178-1334 and 1335-1657).


AA1178
17.3 kDa
AA1335
35.4 kDa
AA1657

helices, ß-strands and random coils. It was suggested that there is a hypothetical DH domain (amino acids 1111 to 1335) and a PH domain (amino acid 1395 to 1515) within the DHR-2 domain (*4*). The hypothetical DH domain within DHR-2 was based on the fact that the typical DH domains of Dbl-family GEFs are also comprised of • -helices with an average size of approximate 200 amino acids. However, the sequence similarity between the DH domains and DHR-2n is quite low and it was impossible to convincingly align the DH domains of Dbl-GEFs with DHR-2n. Moreover, the hypothetical DH or PH domains of DHR-2 failed to exhibit GEF activity towards Rac when they were expressed alone in *E.coli*. This led to the suggestion that the entire DHR-2 domain is essential for Rac activation. However, a construct containing the C-terminal 150 amino acids of DHR-2 had not been previously examined for GEF activity. During our attempt to establish a stable functional sub-domain of DHR-2, we prepared a construct comprised of the C-terminal 150 amino acids of DHR-2 (designated as DHR-2c in Figure 2.9). DHR-2c and a construct comprised on the amino-terminal portions of DHR-2 (DHR-2n) were cloned into the pET28a plasmid and both constructs were expressed in BL-21 DE3 cells. The recombinant proteins were purified as described in the Methods section. Both of the sub-domains were tested for their ability to activate Rac *in vitro.* Mant-exchange assays showed that the DHR-2n region was unable to activate Rac, as previously reported (*4*). However, to our surprise, the DHR-2c sub-domain showed high GEF activity toward Rac (Figure 2.10).

Our results demonstrated that the hypothetical DH domain bears no functional similarity to the classical DH domain of Dbl-GEFs, but rather the C-terminal region of DHR-2 contains the GEF activity. In particular, amino acids 1515 to 1657 were shown to be essential for GEF activity. We also tried to express both the regions 1335-1515

Figure 2.9 Schematic representations of the various DOCK180 and DHR-2 constructs that were used to assay *in vivo* and *in vitro* Rac-GEF activity.

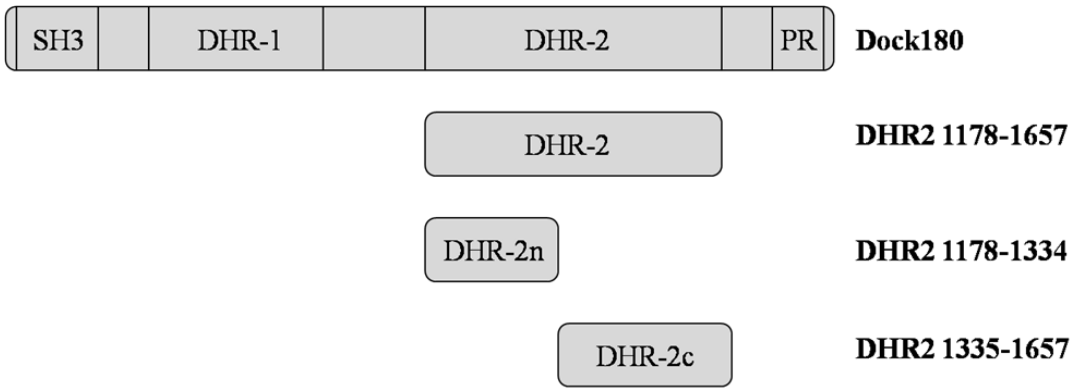
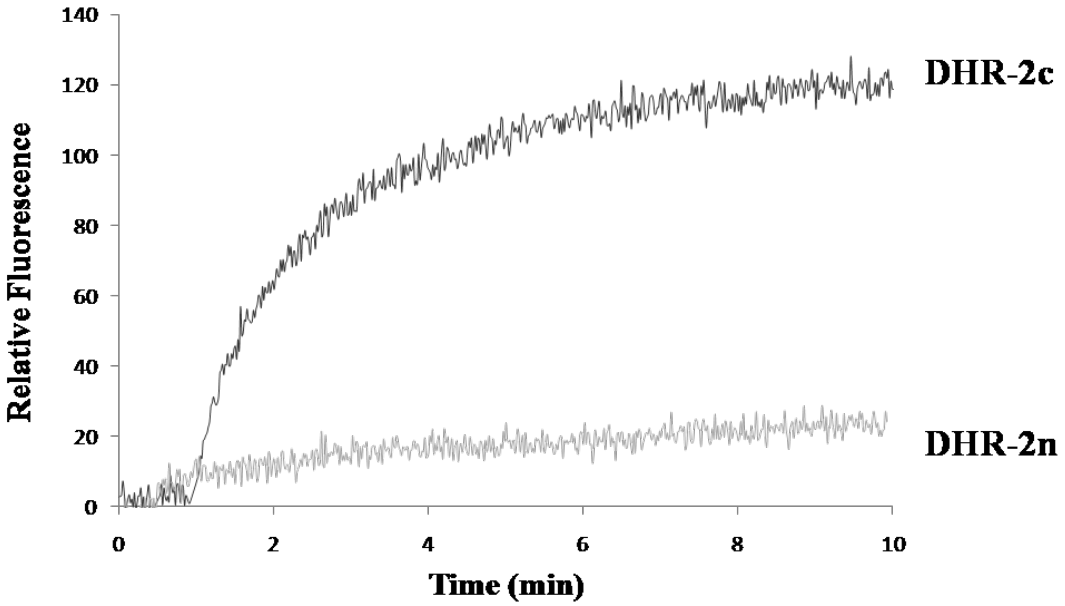

SH3
DHR-1
DHR-2
PR
Dock180
DHR-2
DHR2 1178-1657
DHR-2n
DHR2 1178-1334
DHR-2c
DHR2 1335-1657

Figure 2.10 DHR-2c activates Rac *in vitro*. 600 nM DHR-2c and DHR-2n were incubated with 600nM Rac and 1 μM mant-GDP. DHR-2c showed GEF activity towards Rac, similar to DHR-2, while DHR-2n did not show any ability to activate Rac.


Relative Fluorescence
140
120
100
80
60
40
20
0
0
2
4
6
8
10
Time (min)
DHR-2c
DHR-2n

and 1516-1657. Neither of these regions showed GEF activity toward Rac. Very recently, the X-ray structure of a complex between Zizimin1 (Dock9) and Cdc42 was determined by Barford's group (*5*). Zizimin1 is in the same superfamily with Dock180 and its secondary structure is similar to Dock180 and Zizimin1. Because of these similarities, the mechanisms for the GEF activities of Dock180 and Zizimin1 might be similar. The tertiary structure of the Zizimin1-Cdc42 complex shows that the region corresponding to DHR-2n is not involved in binding Cdc42, consistent with our finding that DHR-2n does not contribute to Rac activation (*5*). The actual contact regions between Zizimin1 and Cdc42 align with several areas within DHR-2c, involving residues 1335-1515 and residues 1516-1657. The detailed interactions will be presented in Chapter 3.

2.3.3 DHR-2c shows similar GEF activity as the DHR-2 domain

The truncated GEF sub-domain of Dock180 (DHR-2c) that will be described throughout the remainder of this study is comprised of amino acids 1335 to 1657. The size of DHR-2c is approximate 37 kDa which is nearly 200 amino acids shorter than the original DHR-2 domain. DHR-2c is highly active as a Rac-GEF. However, a number of questions existed whether this construct fully accounts for the Rac-GEF capability of Dock180, and if so, how it functions as a GEF.

DHR-2c can be well expressed in BL-21 DE3 cells using the pET-28a plasmid, with a yield that is significantly higher than that for the DHR-2 domain. Approximately 15 mg of DHR-2c protein can be obtained from a 2 liter culture compared to 5 mg of protein for DHR-2. DHR-2c can be further purified by a series of chromatography steps starting with nickel-affinity chromatography, followed by ion-

exchange and gel-filtration chromatography. SDS-PAGE showed that DHR-2c is stable in low salt solution, i.e. at 100 mM NaCl (Figure 2.11). Under these conditions, the majority of the DHR-2c exists as a monomer and is better than 95% pure. Purified DHR-2c runs as a doublet on SDS PAGE, perhaps as a result of some limited digestion (also, see Appendix).

In order to compare the GEF activity of DHR-2c with DHR-2, we needed a purified preparation of recombinant DHR-2. We found that gradient elution from a Nickel-affinity chromatography with imidazole yields a DHR-2 preparation that is ~85% pure.

Mant-nucleotide exchange assays (Figure 2.12) showed that, when assaying approximately equal amounts of DHR-2 and DHR-2c, the nucleotide-exchange rates of Rac were nearly identical, whereas, DHR-2n showed no detectable Rac-GEF activity. This indicated that the DHR-2c sub-domain is sufficient for full Rac-GEF activity. The fact that DHR-2n, which is also highly conserved among members of the Dock180 family, has no influence on nucleotide exchange raises the question of what is the function of this region. Recently, based on the structure for the Zizimin1-Cdc42 complex (*5*), it was suggested that the DHR-2n region of Zizimin1 is responsible for the dimerization of Zizimin1 and is important for the function of the DHR-2 domain of Zizimin1, although it does not directly interact with Cdc42. Disruption of the dimerization of DHR-2n through mutagenesis significantly decreased the GEF activity of the DHR-2 domain of Zizimin1. However, base on our results, the DHR-2n construct from Dock180 does not have such a function. First, the DHR-2 domain does not undergo dimerization. While DHR-2 has a tendency to aggregate, the non-aggregated portion remains as a monomer as indicated by gel-filtration

Figure 2.11 DHR-2c is stable in low salt solution. SDS PAGE showed that most of the DHR-2c eluted as a monomer through gel-filtration column, with a small amount being aggregated.

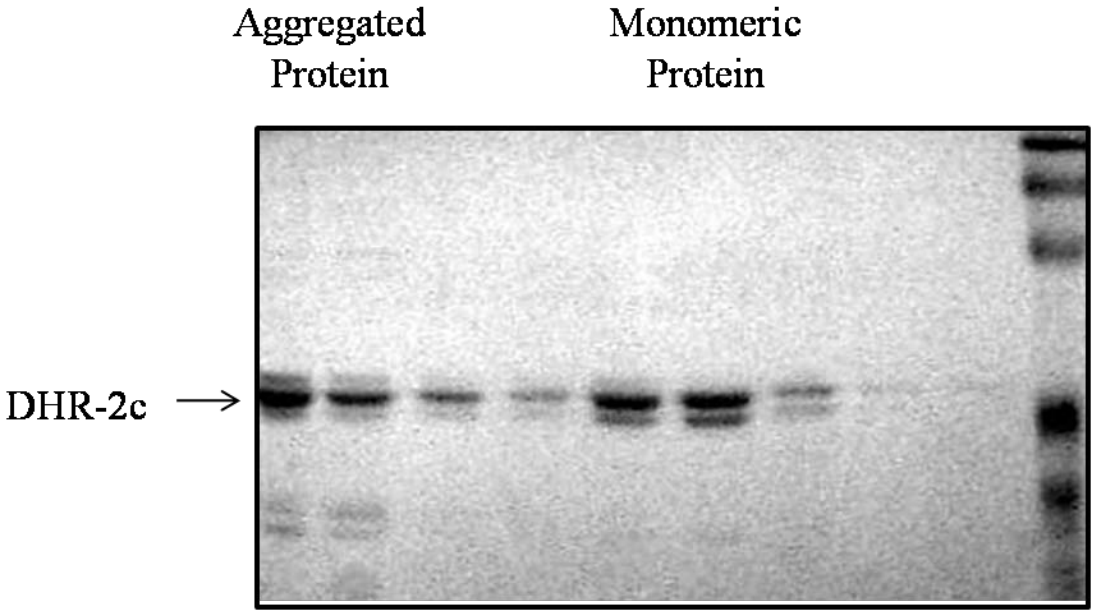

Aggregated Protein
Monomeric Protein
DHR-2c

Figure 2.12 The DHR-2c and DHR-2 domain shows similar GEF activity toward Rac. Equal concentrations (600 nM) of DHR-2c (red curve) and DHR-2 (black curve) were mixed with Rac (600 nM) and mant-GDP (1 μM). The initial reaction rate and the saturation level were approximately same for both

DHIR-2c
DHIR-2
Relative Fluorescence
Time (min)
0
20
40
60
80
100
120
140
0
2
4
6
8
10

chromatography. Second, the absence of the amino-terminal portion of the DHR-2 domain (corresponding to DHR-2n) does not affect the GEF activity of DHR-2c. One possibility is that DHR-2n may contribute to the auto-inhibition of Dock180 by providing the binding area for the SH-3 domain, with this interaction blocking access to the binding site for Rac. We hope to test this possible mechanism in the near future.

2.3.4 DHR-2c showed high specific GEF activity toward Rac

Similar to the case for the DHR-2 domain, DHR-2c shows GEF activity specifically toward Rac. DHR-2c prefers to bind to the nucleotide-free form Rac instead of the GDP- or GTP-bound forms of the GTPase (Figure 2.13)

The structure of DHR-2c is expected to differ from those of the DH domains of Dbl-family GEFs, i.e. based on the known structure of Zizimin1 and Cdc42, we assume that there are two possible mechanisms used by DHR-2c to catalyze nucleotide exchange. The intrinsic nucleotide exchange of GDP for mant-GDP is slow (Pathway I). One possible mechanism is that, when DHR-2c is present, it first forms a complex with Rac-GDP, which triggers the removal of Mg^{2+} and increases the off-rate for the dissociation of GDP. The nucleotide-free Rac complex then binds to mant-GDP which can be detected by monitoring the increase in mant-fluorescence. DHR-2c then dissociates from the Rac-mant-GDP and goes on to target a new Rac molecule (Pathway II-1). However, there is a second possibility, namely, that prior to the dissociation of GDP from Rac (i.e. when Rac is bound to DHR-2c), a second nucleotide (i.e. mant-GDP) binds and helps trigger the release of the GDP molecule (Pathway II-2). In either case, the total amount of Rac which binds to GDP or mant-GDP depends on the initial concentration of GDP and mant-GDP in solution. DHR-2c

Figure 2.13 DHR-2c binds tightly to nucleotide-free Rac. Similar to full-length DHR-2, the binding of DHR-2c to nucleotide-free Rac is much stronger than to either GDP-bound or GTP-bound Rac.

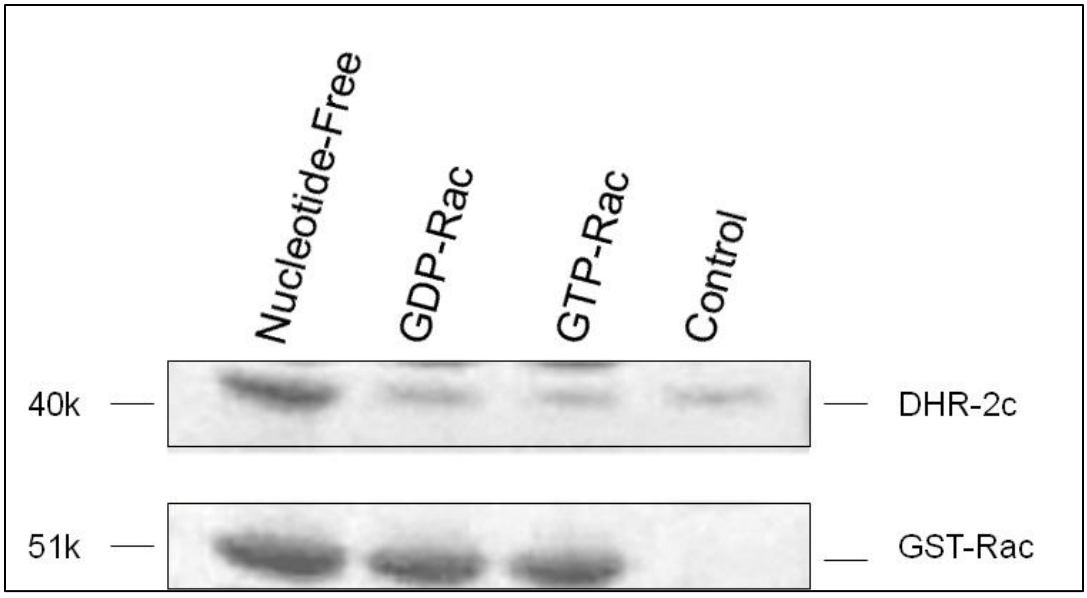
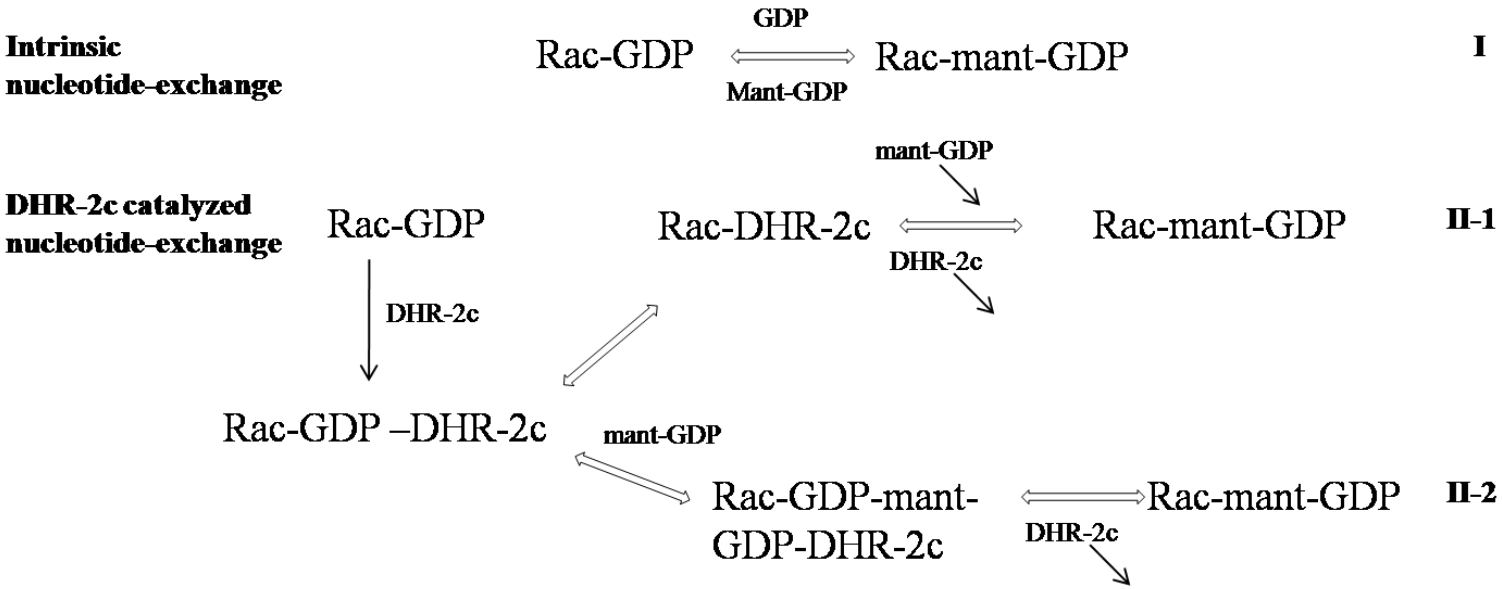

Nucleotide-Free
GDP-Rac
GTP-Rac
Control
40k
DHR-2c
51k
GST-Rac

Figure 2.14 The pathway representing intrinsic nucleotide exchange (Pathway I) and the two possible pathways for DHR-2c-catalyzed nucleotide exchange (Pathway II-1 and II-2).


Intrinsic nucleotide-exchange
GDP
Rac-GDP
Rac-mant-GDP
Mant-GDP
I
DHR-2c catalyzed nucleotide-exchange
mant-GDP
Rac-GDP
Rac-DHR-2c
Rac-mant-GDP
DHR-2c
II-1
DHR-2c
Rac-GDP –DHR-2c
mant-GDP
Rac-GDP-mant-GDP-DHR-2c
Rac-mant-GDP
DHR-2c
II-2

is able to act in a catalytic fashion to stimulate nucleotide exchange on Rac. Figure 2.15 shows that, when equivalent amounts of Rac-GDP and mant-GDP were mixed with different concentrations of DHR-2c (while maintaining Rac-GDP in excess), increasing the concentration of DHR-2c only affects the initial rate for nucleotide-tide exchange and not the total amount of Rac that is activated.

The nucleotide exchange of GDP for mant-GDP on Rac, as catalyzed by DHR-2c, is reversible (Pathway II in Fig 2.14). However, if a substantial excess of GDP or mant-GDP is present in solution, Pathway II can be considered to be irreversible. Under such conditions, it is possible to determine turnover rates for DHR-2c-catalyzed nucleotide exchange on Rac. Specifically, this can be done by measuring the fluorescence increase that accompanies the exchange of GDP for mant-GDP on Rac, under conditions of excess mant-GDP, or by monitoring the loss of mant-fluorescence when exchanging mant-GDP for GDP (i.e. the reverse reaction) when GDP is used in high excess. However, there are some technical challenges when using the former approach because of the background fluorescence contributed by free mant-GDP, and so for many of our studies we chose to monitor the changes in mant-fluorescence that accompany the exchange of mant-GDP for GDP. As shown in Figure 2.16, when a solution containing Rac-mant-GDP was incubated with a large excess of GDP, the rate of nucleotide exchange was slow. Addition of DHR-2c (at concentrations much lower than Rac-mant-GDP) significantly accelerated the reaction. After mixing 20 nM DHR-2c and several different concentrations of Rac-mant-GDP (200 nM to 4 μM) with 100 μM GDP, we obtained a series of fluorescent traces and calculated their corresponding initial rates (v). Using the Michaelis-Menton equation, we plotted $1/v$ (normalized to the DHR-2c concentration) against 1/[Rac-mant-GDP] (Figure 2.17). We obtained a Km (half-maximal saturation) value of at 3.3 μM and a maximal rate of dissociation

Figure 2.15 DHR-2c activates Rac catalytically. Fluorescence tracings obtained when incubating 3 μM Rac with decreasing concentrations of DHR-2c, as indicated, in the presence of 1 μM Mant-GDP. The control trace was obtained when measuring mant-GDP exchange in the absence of DHR-2c.

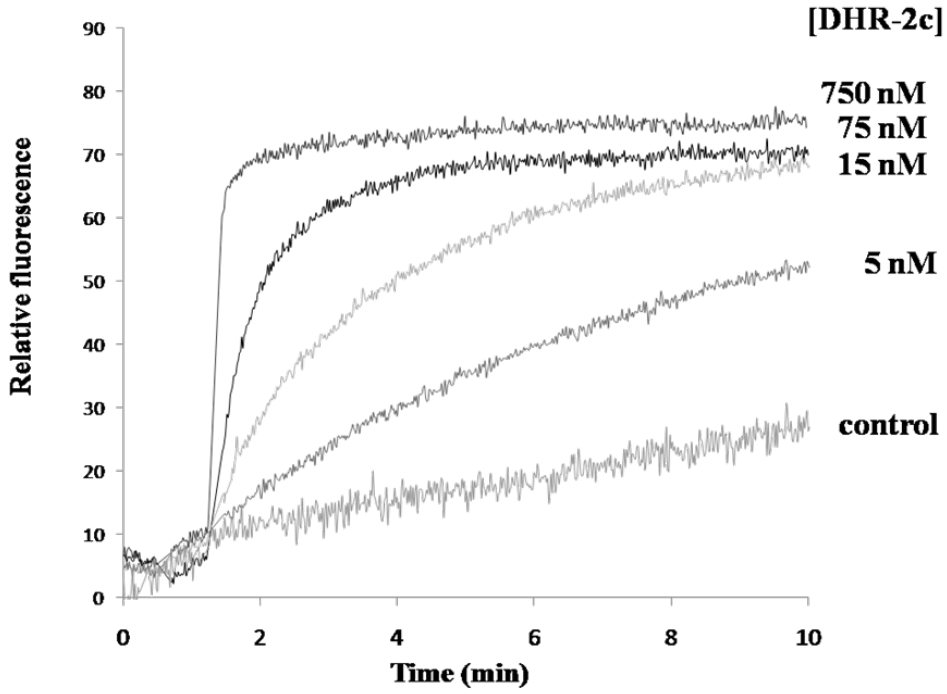

[DHR-2c]
750 nM
75 nM
15 nM
5 nM
control
Relative fluorescence
Time (min)
0
10
20
30
40
50
60
70
80
90
0
2
4
6
8
10

Figure 2.16 Nucleotide exchange was detected by monitoring the fluorescent decrease upon mixing Rac-mant-GDP (600 nM) with DHR-2c (60 nM) and excess GTP (100 μM)

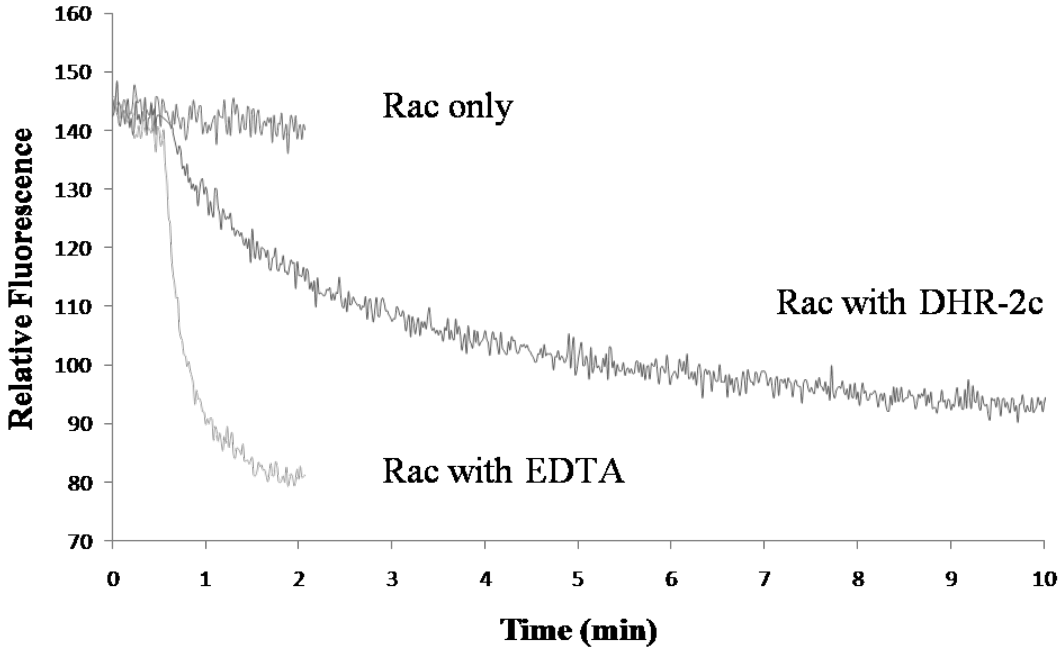
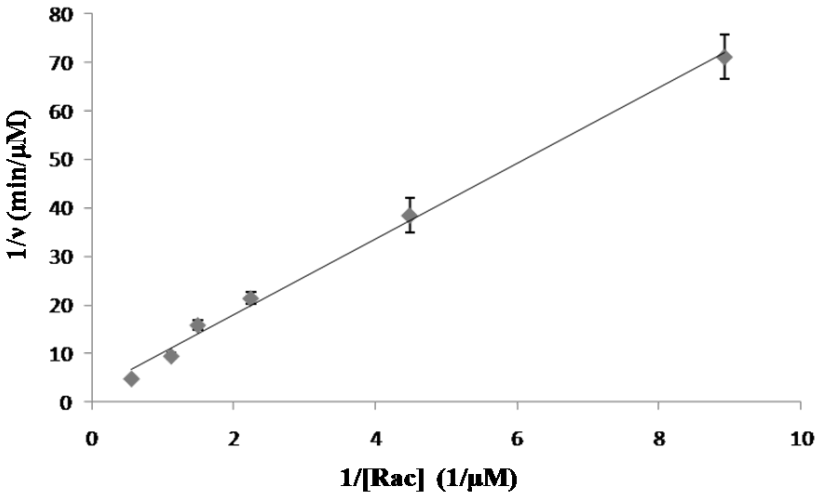

160
150
140
130
120
110
100
90
80
70
Relative Fluorescence
Rac only
Rac with DHR-2c
Rac with EDTA
0
1
2
3
4
5
6
7
8
9
10
Time (min)

Figure 2.17 The dependence of initial velocity (•) on the concentration of Rac-Mant-GDP. The reaction contained initially 20 nM DHR-2c, 100 μM GTP and various concentrations of Rac-Mant-Rac. Initial velocity was calculated by observing the decrease in fluorescence at 440 nm. 1/• was plotted against 1/[Rac] and fitted to the Michaelis-Menten equation, yielding a Km of 3.3±0.2 μM and a k_{cat} value of 19.8±3.2 /min.


80
70
60
50
40
30
20
10
0
1/v (min/µM)
0
2
4
6
8
10
1/[Rac] (1/µM)

for GDP (*k*cat) of 19.8/min. This means that DHR-2c can catalyze the exchange the nucleotide on 20 molecules of Rac per minute.

We also were interested in determining the intrinsic rate of nucleotide dissociation from Rac. Fluorescent mant-exchange assays showed that nucleotide-exchange on Rac in the absence of DHR-2c is very slow. The intrinsic nucleotide dissociation constant of Rac, $k_{\text{-mant-GDP,}}$ is approximately 0.0166/min. By comparing the k_{cat} and $k_{\text{-mant-GDP}}$ values, we concluded that the GDP-dissociation rate from Rac is increased ~1000 fold when DHR-2c is presented in solution. These results demonstrated that DHR-2c was highly active on Rac *in vitro.*

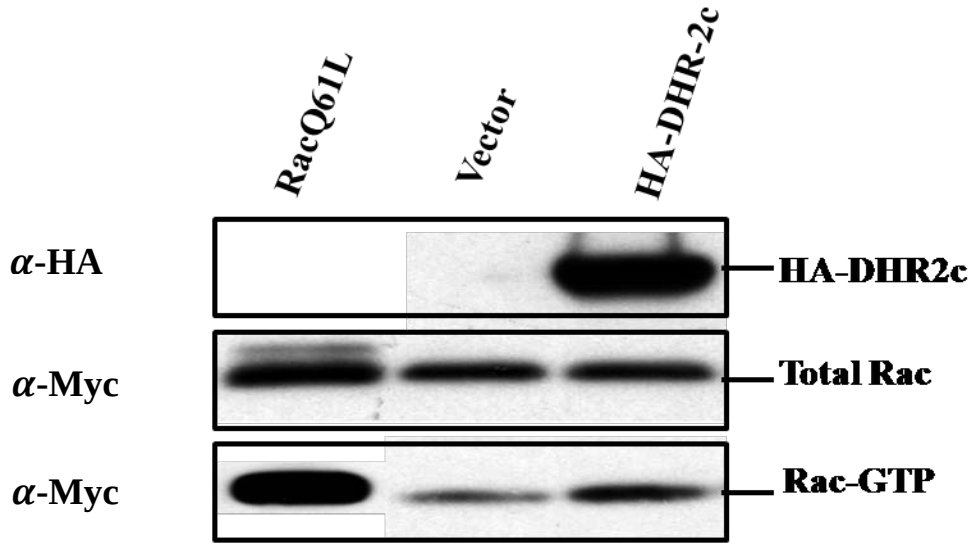
2.3.5 DHR-2c activates Rac *in vivo*

There has been are some controversy about whether the DHR-2 domain is sufficient to activate Rac *in vivo* or if it requires Elmo, as has been suggested for the full-length Dock180 (*6*). To establish whether Rac can be activated *in vivo* by DHR-2c, Cos-7 cells were transiently transfected with plasmids encoding HA-DHR-2c and Myc-Rac and then plated overnight. Figure 2.18 shows the co-localization of DHR-2c and Rac at the plasma membrane and the formation of membrane ruffles due to changes in actin cytoskeletal organization. This demonstrated that the DHR-2c domain can activate Rac in Cos-7 cells. To further confirm that Rac is activated by DHR-2c in cells. Lysates were prepared and incubated with GST-PBD preloaded onto glutathione beads. Figure 2-19 shows that greater amounts of Rac-GTP can be precipitated by GST-PBD from cells expressing DHR-2c compared to control cells.

Figure 2.18 Rac and DHR-2c co-localize in membrane ruffles. Indirect immunofluoresence of Cos-7 cells cotransfected with myc-Rac and HA- DHR-2c.

HA-DHR-2c
Myc-Rac$_{wt}$

Figure 2.19 PBD assay demonstrates activation of Rac in vivo by DHR-2c. GTP-bound Rac was affinity precipitated with PBD beads from Cos-7 cell lysates that had been transiently transfected with vector, myc-Rac-WT, myc-RacQ61L, or RacWT+HA-tagged DHR-2c. Bottom row of immunoblot shows the relative amounts of activated Rac under described conditions.


RacQ61L
Vector
HA-DHR-2c
α-HA
HA-DHR2c
α-Myc
Total Rac
α-Myc
Rac-GTP

2.4 Discussion

Since Dock180 was discovered in 1992, a significant amount of effort has been put into the investigation and characterization of this new Rho-GTPase GEF and its other family members. As a Rac-specific GEF, Dock180 was found to be involved in a number of biological pathways by regulating the activity of Rac. It was reported to be able to trigger GDP-GTP exchange on Rac both *in vitro* and *in vivo.* The functional domain of Dock180 is called the DHR-2 domain and is fully responsible for the GEF activity of Dock180. All the DHR-2 domains of Dock180 family members have been found to activate Rac, Cdc42 or both of these GTPases. None has been shown to activate Rho. The reason for this is not yet clear.

The DHR-2 domain is conserved throughout the Dock180 family; however, the similarity between different sub-domains is quite low. Recently, the structure of the DHR-2 domain of one family member, Dock9 (Zizimin1), was reported (*5*). However, based on the structural information obtained for Dock9, it is still very hard to predict the structure of Dock180 and the mechanism by which it activates Rac, because of the low sequence similarity (~19%) between Dock9 and Dock180. Several groups have been working on functional studies of the DHR-2 domain. Because of its low expression levels in *E.coli*, *in vitro* data has been difficult to obtain.

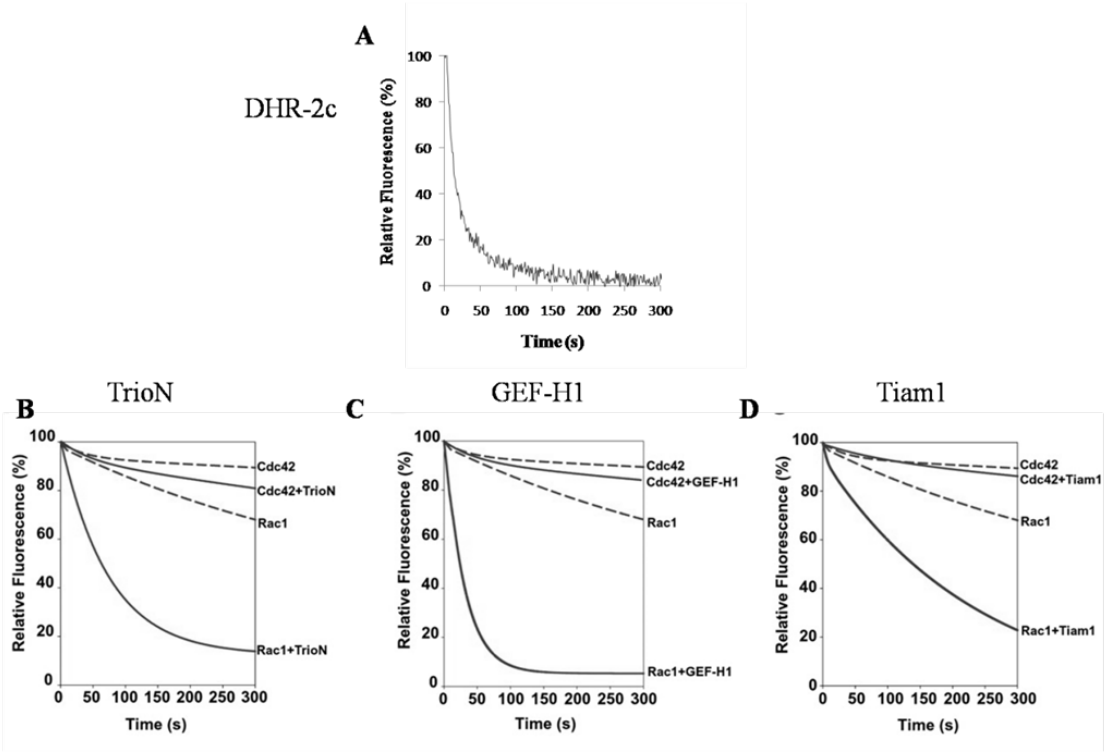
In our laboratory, we discovered a limit DHR-2 domain (DHR-2c), which is shorter by 200 amino-acids compared to the original DHR-2 domain. It still exhibits full GEF activity toward Rac. Based on the published data, as well as our results, the DHR-2 domain of Dock180 is the most active domain of the family. The *k*cat of DHR-2c is ~20/min which is 1000-fold greater than the intrinsic nucleotide dissociation rate of Rac. Even when compared with Dock9, a Cdc42-GEF, DHR-2c is 30-fold faster than the DHR-2 domain of Dock9. The GEF activities of the DH domains of classic

Dbl-GEFs are variable. The activities of some Dbl-GEFs can barely be detected *in vitro.* Based on the available literature, DHR-2c shows higher GEF activity than the Dbl-GEFs (Figure 2.20). In Figure 2.20, the GEF activity of DHR-2c is compared to three members of the Dbl-GEF family that are specific for Rac. GEF-H1 is the most active GEF for Rac among the three Dbl-GEFs, with DHR-2c showing even greater GEF activity than GEF-H1. Why Dock180 shows so high activity toward Rac is not well understood. The main function of Dock180 is related with Rac-mediated phagocytosis. Because the process of phagocytosis is only around 5 to 10 minutes after the 'eat-me' signals are sent out by apoptotic cells, high-efficient Dock180 can quickly activate Rac in the cell. Rac-mediated ruffle formation can surround the apoptotic cell and prevent the poisonous extent to be released outside of cells.

The DHR-2c domain of Dock180 can activate Rac both *in vitro* and *in vivo.* However, the same region from other family members can not fully activate their corresponding GTPases. The DHR-2c region of Dock9 showed much weaker activity toward Cdc42 compared to the full-length DHR-2 domain because the DHR-2n region is reported to be important for the dimerization of Dock9 (*5*). We cloned the same regions from Dock7 and Dock11 and found that neither showed GEF activity toward Cdc42 or Rac. Thus, DHR-2c of Dock180 appears to be a unique region in its ability to exhibit high GEF activity.

As a GEF for Rac, Dock180 is tightly regulated in cells. Dock180 participates in a number of Rac-dependant signaling pathways and is recognized as one of the main activators of Rac. As mentioned above, there are two large superfamilies of GEFs containing more than 70 members each that activate Rho GTPases. This raises the common question of why are so many Rho-GEFs needed for the same GTPase? Or put another way, why is the Dock180 family needed when there is already a large

Figure 2.20 DHR-2c showed higher GEF activity than the DH domain of Dbl-GEFs. A) 200 nM Rac1 loaded with mant-GDP was incubated with 200 nM DHR-2c at room temperature in an exchange buffer containing 20 mM Hepes (pH 8.0), 5 mM $MgCl_2$ and 0.5 mM GTP. DHR-2c caused a faster decrease in mant-fluorescence compare to the DH domains of TrioN, GEF-H1 and Tiam1 as shown in Figures B-D, respectively. Figures B-D were taken from Zheng and colleagues' paper (*1*).


A
DHR-2c
Relative Fluorescence (%)
Time (s)
B
TrioN
Cdc42
Cdc42+TrioN
Rac1
Rac1+TrioN
C
GEF-H1
Cdc42
Cdc42+GEF-H1
Rac1
Rac1+GEF-H1
D
Tiam1
Cdc42
Cdc42+Tiam1
Rac1
Rac1+Tiam1

family of Dbl-GEFs? Are their significant differences in their mechanisms of action? How do Rho-GTPases select a particular GEF for in a specific signaling pathway? In the next chapter, some of our recent work will be presented that considers the similarities and differences of Rac activation by Dock180 and Tiam1, which are representative members of the Dock180 and Dbl-GEF families.

REFERENCES

1. Gao, Y., Xing, J., Streuli, M., Leto, T. L., and Zheng, Y. (2001) Trp(56) of rac1 specifies interaction with a subset of guanine nucleotide exchange factors, *J Biol Chem 276*, 47530-47541.
2. Brugnera, E., Haney, L., Grimsley, C., Lu, M., Walk, S. F., Tosello-Trampont, A. C., Macara, I. G., Madhani, H., Fink, G. R., and Ravichandran, K. S. (2002) Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex, *Nat Cell Biol 4*, 574-582.
3. Lu, M., Kinchen, J. M., Rossman, K. L., Grimsley, C., Hall, M., Sondek, J., Hengartner, M. O., Yajnik, V., and Ravichandran, K. S. (2005) A Steric-inhibition model for regulation of nucleotide exchange via the Dock180 family of GEFs, *Curr Biol 15*, 371-377.
4. Cote, J. F., and Vuori, K. (2002) Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity, *J Cell Sci 115*, 4901-4913.
5. Yang, J., Zhang, Z., Roe, S. M., Marshall, C. J., and Barford, D. (2009) Activation of Rho GTPases by DOCK exchange factors is mediated by a nucleotide sensor, *Science 325*, 1398-1402.
6. Grimsley, C. M., Kinchen, J. M., Tosello-Trampont, A. C., Brugnera, E., Haney, L. B., Lu, M., Chen, Q., Klingele, D., Hengartner, M. O., and Ravichandran, K. S. (2004) Dock180 and ELMO1 proteins cooperate to promote evolutionarily conserved Rac-dependent cell migration, *J Biol Chem 279*, 6087-6097.

CHAPTER 3

RECOGNITION OF SPECIFIC BINDING BETWEEN RAC AND DHR-2C

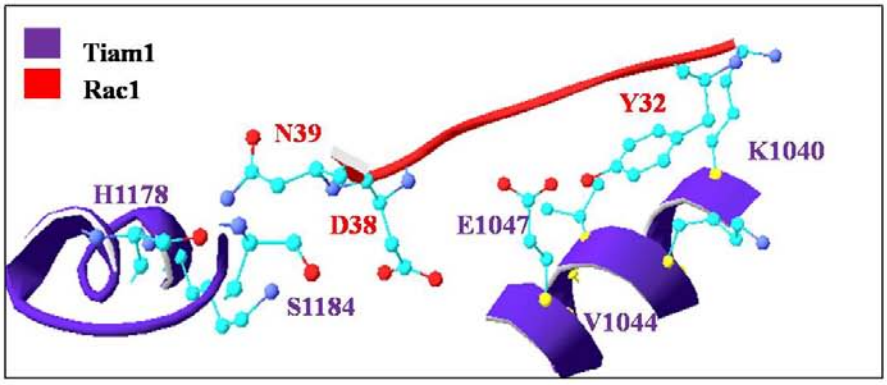
3.1 Introduction

Members of the two large families of Rho-guanine nucleotide exchange factors (GEFs), the classical Dbl-GEFs and the more recently discovered Dock180 family, play vital roles in cellular signal transduction. However, when comparing their primary sequences and three-dimensional structures, it is clear that the Dbl-GEFs and Dock180 family members are quite distinctive from each other. Since the x-ray structure for the complex formed between Tiam1 and Rac was first solved in 2000 by Sondek's laboratory (*1*), a number of structures of Dbl-GEF have been determined and the mechanism by which they activate Rho GTPase activation by Dbl-GEFs has been established based on the structural information. The DH-domains of all the known Dbl-GEFs comprise • -helices only, and their contact regions with Rho GTPases are very similar.

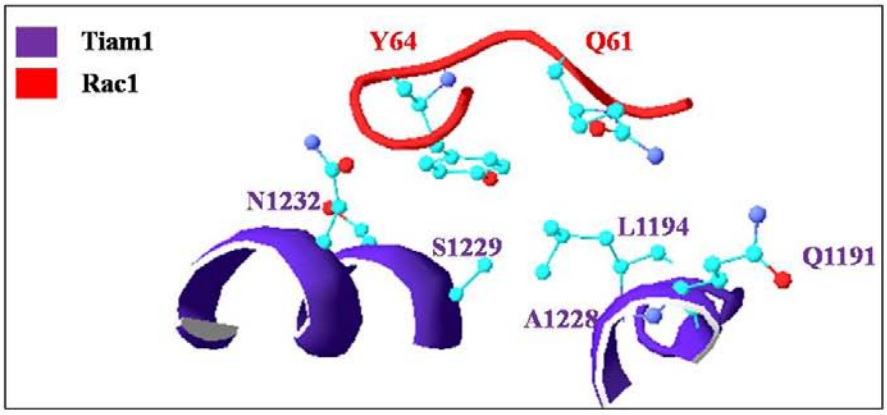
Tiam1 is a representative of the Dbl-GEF family, and a good deal of work has been done to investigate its interaction with and activation of Rac. It has been reported that the main contact regions for Tiam1 on Rac are located in the switch I and switch II regions as shown in Figure 3.1. In the switch I region, mutagenesis studies have shown that the Cdc42 (Tyr32Ala) mutant retained the ability to bind to Tiam1 but was unable to be activated by the GEF, while the Cdc42 (Asp38Ala) and Cdc42 (Asn39Ala) mutants lost their ability to bind Tiam1. The Cdc42 (Gln61Leu) and Cdc42 (Tyr64Ala) mutants, which contain substitution within switch II, were also found to be unable to couple to the DH domain of Tiam1(*2*). These results indicated

Figure 3.1 The main contact areas of Rac with Tiam1 are located in switch I, switch II and the ß2-ß3 region. (A) Cdc42 (Tyr32Ala) mutant retained the ability to bind to Tiam1 but was unable to be activated by the GEF, while the Cdc42 (Asp38Ala) and Cdc42 (Asn39Ala) mutants lost their ability to bind Tiam1. (B) Single-substitutions at Glu61 and Tyr64 all significantly affect the Rac binding and the activation by Tiam1. (C) Trp56 of Rac is the only residue which is responsible for the Dbl-GEF specific recognition.

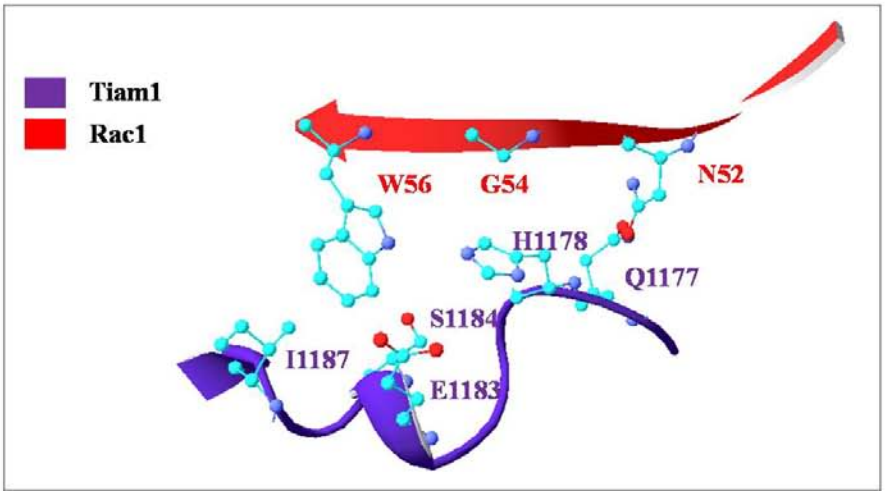
A


Tiam1
Rac1
N39
Y32
K1040
H1178
D38
E1047
S1184
V1044

B


Tiam1
Rac1
Y64
Q61
N1232
L1194
Q1191
S1229
A1228

C


Tiam1
Rac1
W56
G54
N52
H1178
Q1177
S1184
I1187
E1183

that the switch I and switch II regions of Rac are both important for Tiam1 binding and activation.

In addition to the switch I and switch II regions, there is another key residue, Trp56, which is located within the •2-•3 region of Rac. It was reported to not only be critical for the Tiam1 interaction, but it is the only residue that serves to ensure that Rac distinguishes Tiam1 from a Cdc42-specific GEF i.e. Intersectin. Changing this tryptophan to phenylalanine (i.e. the corresponding residue in Cdc42) enables Rac to be recognized by Cdc42-GEFs (*2*).

The activation mechanism of Rac/Cdc42 by Dock180-GEFs is still not well understood. Only very recently has information become available for the DHR-2 domain of the Dock180-GEFs. Yang and colleagues reported the structure of the complex formed between the DHR-2 domain of Zizimin1 (Dock9) and Cdc42 (*3*). Some information regarding the sites of contact between Zizimin1 and Cdc42 can be extracted from the 3D structure of the complex. However, those residues that are really important for the interaction are still not known.

3.2 Methods

Plasmids. Rac, Cdc42 and DHR-2c mutants were generated from plasmids encoding wild-type Rac, Cdc42, and DHR-2c, using site-directed mutagenesis kits purchased from Stratagene.

Protein Expression and Purification. Single colonies of *E.coli* BL21 (DE3) containing target plasmids were inoculated in 10 ml of LB media with 50 µg/ml kanamysin/Carbenicillin (RPI) and cultured overnight at 37°C. These small cultures

were subsequently used to inoculate 1 L LB media with 50 μg/ml kanamysin/ carbenicillin in a shaking incubator at 37°C. The large-scale cultures were incubated to a density of OD_{600}=0.6 and induced by IPTG (RPI) (final concentration = 200 μM) at room temperature overnight. Bacteria were harvested by centrifugation at 5000 rpm for 10 minutes and the pellets were re-suspended in lysis buffer (20 mM Tris-HCl PH 8.0, 5 mM $MgCl_2$, 500 mM NaCl) with 10 μg/ml leupeptin and 10 μg/ml aprotinin.

For DHR-2c and its mutants, the suspensions were sonicated and the lysates were cleared by centrifugation at 20000 rpm for 30 minutes. The supernatants were collected and incubated with nickel chelate beads (Amersham) for 30 minutes on ice. The beads were washed with lysis buffer containing 40 mM imidazole until no significant protein was detected in the wash buffer. The proteins were eluted with lysis buffer containing 200 mM imidazole, concentrated to 200 μM, and stored at -80°C for further use.

Rac, Cdc42 and their mutants were expressed using similar procedures as described above and the supernatants were collected and applied to a glutathione-sepharose column. Unbound proteins were washed off by lysis buffer and target proteins were eluted using the same buffer with 10 mM glutathione. The eluted proteins were applied to P-10 desalting column to remove glutathione and concentrated to 300 μM.

In vitro Guanine Nucleotide Exchange Assay. All fluorescence measurements were performed using a Varian Ecilpse Fluorescence Spectrophotometer. Samples were stirred continuously and thermostated at 25 °C in HMA buffer (20 mM HEPES pH 8.0, 5 mM $MgCl_2$ and 1 mM NaN_3). *In vitro* GEF assays using mant-GDP as a probe to monitor fluorescent changes accompanying nucleotide exchange were performed on recombinant GTPases. Mant-GDP was added to HMA buffer to a final

concentration of 1 μM. Different concentrations of Rac/ Cdc42 and their mutants were added together with various concentrations of the DHR-2c domain of Dock180 and the DH-PH domain of Tiam1. The Mant-GDP fluorescence changes were monitored using an excitation wavelength of 340 nm and an emission wavelength of 440 nm at 25°C. Each measurement was repeated at least three times.

3.3 Results

3.3.1 Trp 56 of Rac is important for Dock180 binding and activation

As a first step toward investigating the mechanism by which Dock180 activates Rac, we examined the effects of NSC 23766, a small molecular inhibitor of Rac activation. NSC 23766 was originally identified using a 'virtual screen' for Rac inhibitors from the National Cancer Institute Database (*4*). The compound was predicted to dock into the active pocket of Rac, surrounding tryptophan 56 and thereby blocking the interaction of Rac with traditional Dbl-family GEFs like Tiam1. This compound was experimentally tested for its ability to inhibit the actions of Tiam1 and another GEF, LBC toward Rac. The results showed that NSC 23766 was capable of both *in vivo* and *in vitro* inhibition of the Rac-GEF activity of Tiam1 and LBC. However, the inhibition observed *in vitro* was relatively weak.

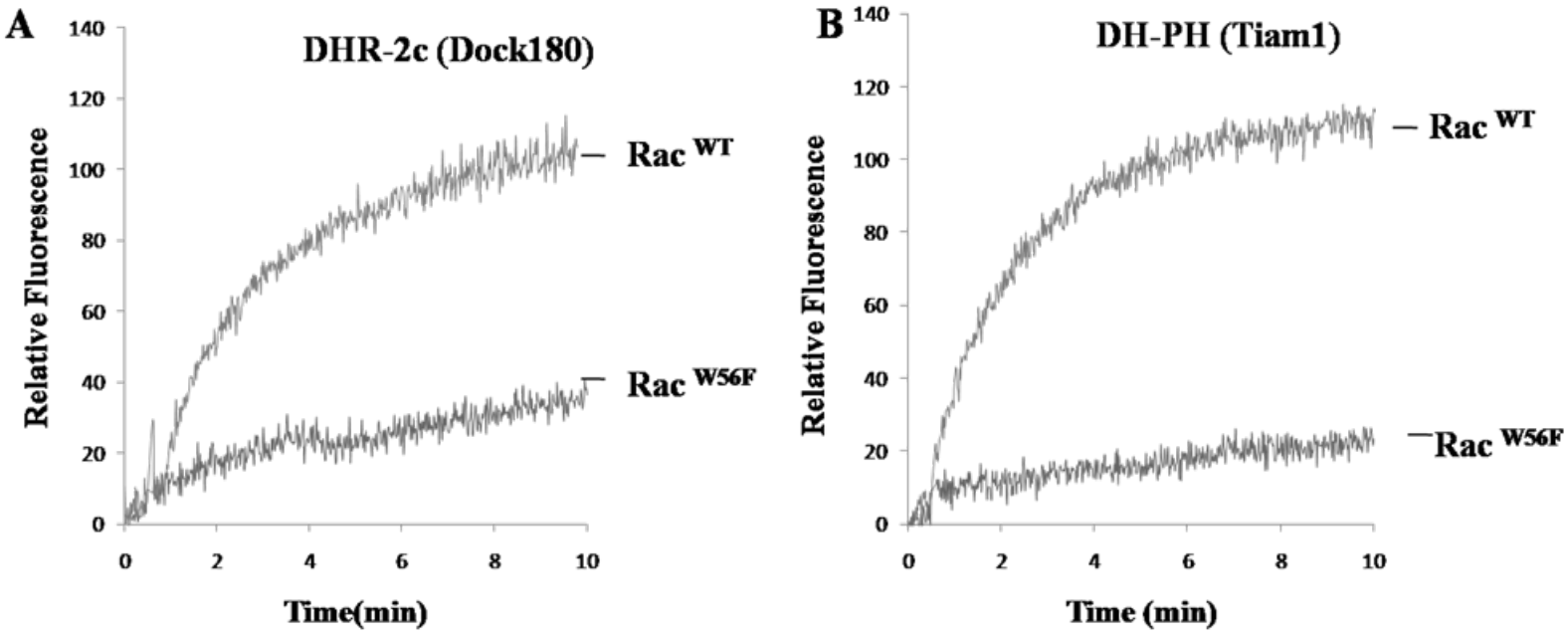
We have tested the inhibitory ability of NSC 23766 toward Dock180. Varying concentrations of NSC 23766 were premixed with Rac and mant-GDP for 10 minutes and then DHR-2c was added to the mixture. The activation of Rac was monitored by tracking changes in mant-fluorescence accompanying nucleotide exchange. The nucleotide exchange rates were much lower when NSC 23766 was present, compared to the control (which lacked the inhibitor). NSC 23766 showed similar inhibitory

ability against the DHR-2c domain as seen against Tiam1. Pull-down assays also showed that NSC 23766 disrupted complex formation between DHR-2c and Rac. However, the inhibition was not complete even when the concentration of NSC 23766 was in 200-fold excess relative to Rac. Overall, our results seemed to indicate that NSC 23766 competes with DHR-2c for the binding to Trp56 which partially blocks the interaction between DHR-2c and Rac.

To further confirm the importance of this tryptophan residue for the binding of Rac to DHR-2c, Trp56 of Rac was changed to phenylalanine (the corresponding residue in Cdc42). Fluorescence exchange assay showed that this single substitution totally abolished Rac activation by DHR-2c (Figure 3.2A) and also eliminated the interaction between Rac and DHR-2c. We also confirmed that nucleotide exchange on this Rac mutant can be triggered by adding EDTA, which means that it is still capable of nucleotide binding. These results demonstrated that Trp56 of Rac is critical for the interaction with both Dock180 and Dbl-family Rac GEFs.

Trp56 is located in the • 2-• 3 loop of Rac. There are some other residues in this region which are also reported to be important for Dbl-GEF recognition. Mutation of Gly54 to alanine makes Rac insensitive to Tiam1. To test whether Gly54 of Rac is also important for DHR-2 binding, we made the same Rac (Gly54Ala) mutant. Fluorescence mant-assays confirmed that nucleotide exchange on this mutant can not be triggered by the DHR-2c domain. These results demonstrated that the ß2-ß3 region of Rac is an important docking area for DHR-2c, similar to the case for DH domains of Dbl-like GEFs.

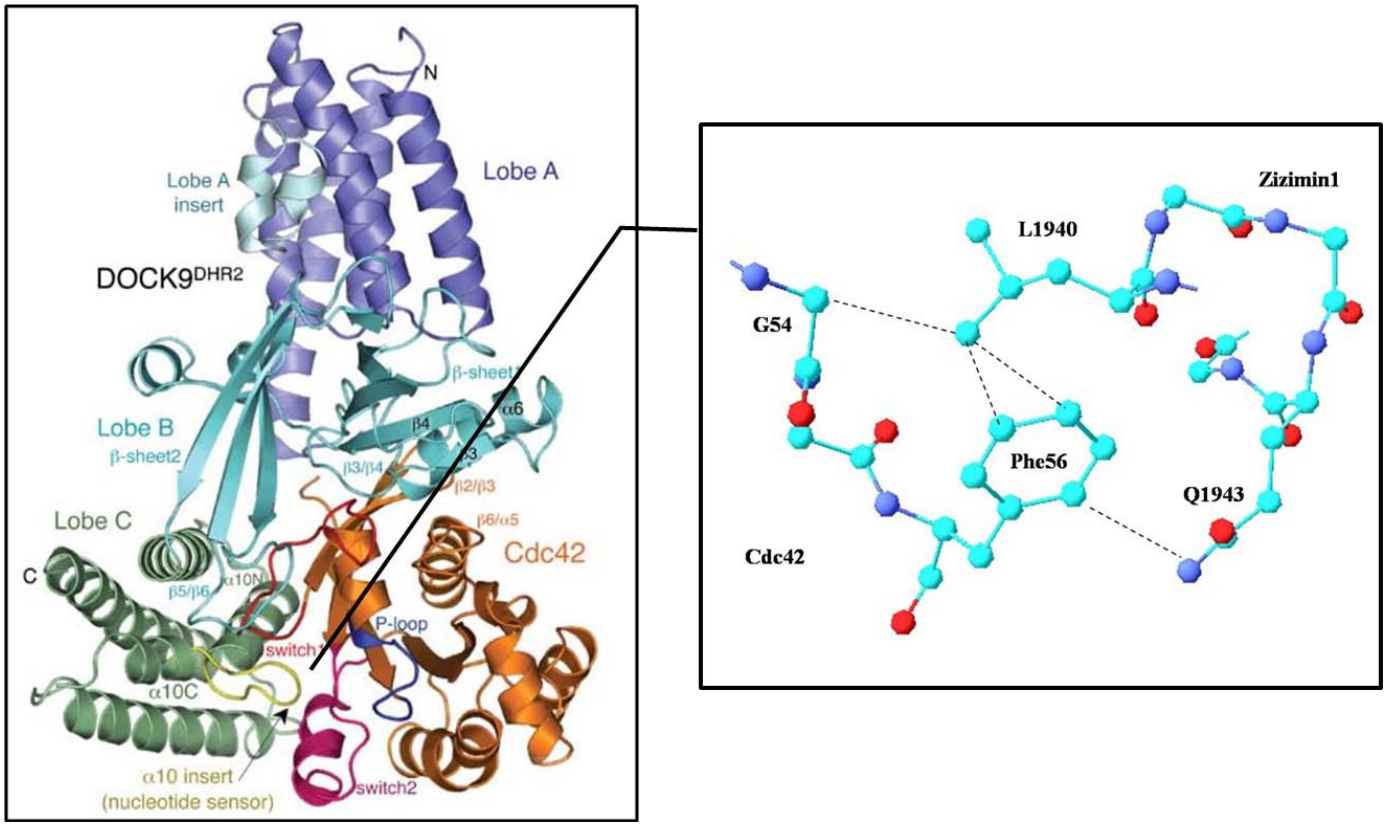
Figure 3.2 Trp56 of Rac is critical for both Dock180 and Tiam1 recognition. 180 nM DHR-2c (A) or 15 μM DH-PH domain of Tiam1(B) were mixed with same concentration (600 nM) of wild-type Rac or the Rac(Trp56Phe) mutant. Rac (Trp56Phe) was not able to respond to either the DHR-2c domain of Dock180 or the DH-PH domain of Tiam1.


A
DHR-2c (Dock180)
Relative Fluorescence
Time(min)
Rac WT
Rac W56F
B
DH-PH (Tiam1)
Relative Fluorescence
Time (min)
Rac WT
Rac W56F

3.3.2 Trp56 of Rac specifically recognizes Met1524 of Dock180

The next question that we were interested in addressing was what are the corresponding residues on DHR-2 which interact with and recognize Gly54 and Trp56 of Rac. The x-ray structure for the Zizimin1-Cdc42 complex provides us with some clues regarding the nature of the Rac-DHR-2 interaction. From the structure (Figure 3.3), the only residues that are close to Gly54 and Phe56 of Cdc42 are Leu1940 and Gln1943 of Zizimin1. In particular, the methyl group of Leu1940 is in close proximity (~3.5 A) to both the benzene ring of Phe56 and the backbone of Gly54. It may form hydrophobic interactions with these two residues. More importantly, this leucine residue is conserved in all Dock D subfamily members (i.e. specific GEFs for Cdc42). We hypothesized that this leucine residue is necessary for specifically recognizing the phenylalanine residue of Cdc42, while Met1524 of Dock180, which is conserved at the same site on other Dock180-GEFs (Figure 3.4), is responsible for Rac-GEF specificity. Based on our prediction, Met1524 of DHR-2c should contact directly Trp56 of Rac, and if we mutated this residue, the interaction between DHR-2c and Rac should be severely influenced. In order to confirm this, we mutated Met1524 of DHR-2c to alanine. Removal of the side chain of methionine significantly alters the GEF activity of DHR-2c. Fluorescence mant-assays showed that the GEF activity of the DHR-2c (Met1524Ala) mutant toward Rac is only about 20% of the Rac-GEF activity of wild- type DHR-2c (Figure 3.5). We then examined whether the loss of the GEF activity is due to the inability of the DHR-2c (Met1524Ala) mutant to bind to Trp56 of Rac. We changed Met1524 to leucine which is the corresponding residue in Zizimin1 (i.e. Leu1940). Although this represented a conservative substitution, the resultant DHR-2c (Met1524Leu) mutant was incapable of activating Rac (Figure 3.5), similar to the case for the DHR-2c (Met1524Ala) mutant. This told us that Rac is very

Figure 3.3 Structure of DHR-2 (Zizimin1)-Cdc42 complex and the detail binding area between the ß3 region of Rac and the DHR-2c domain of Zizimin1. Leu1940 of Zizimin1 is in close proximity (~3.5 A) to the benzene ring of Phe56 and the backbone of Gly54. Q1943 of Zizimin1 is also close to Phe56 of Rac.



Yang *et.al. Science 2009*

Figure 3.4 Alignment the region of DHR-2 which binds to the • 2-• 3 region of Rac. Met1524 of Dock180 is conserved in all Dock180-family members except in the Cdc42-specific GEFs (leucine is present at the corresponding position in Cdc42) .

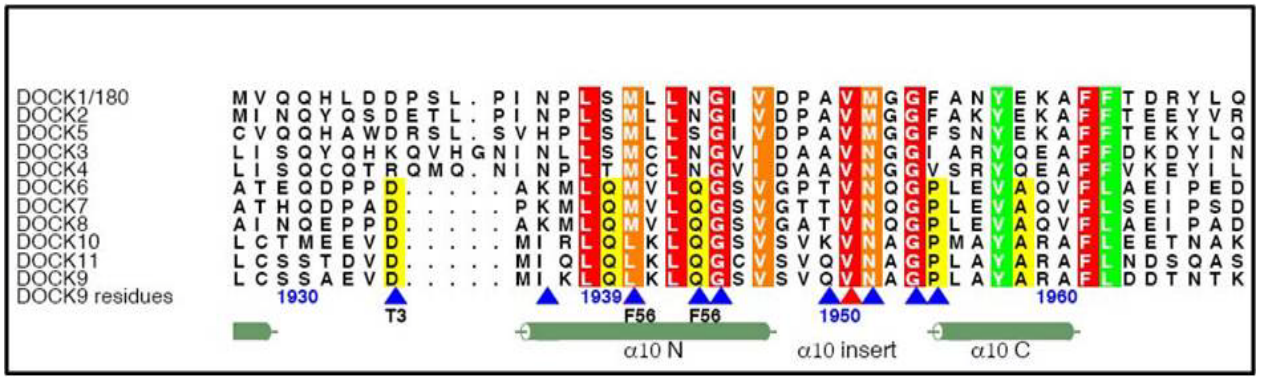
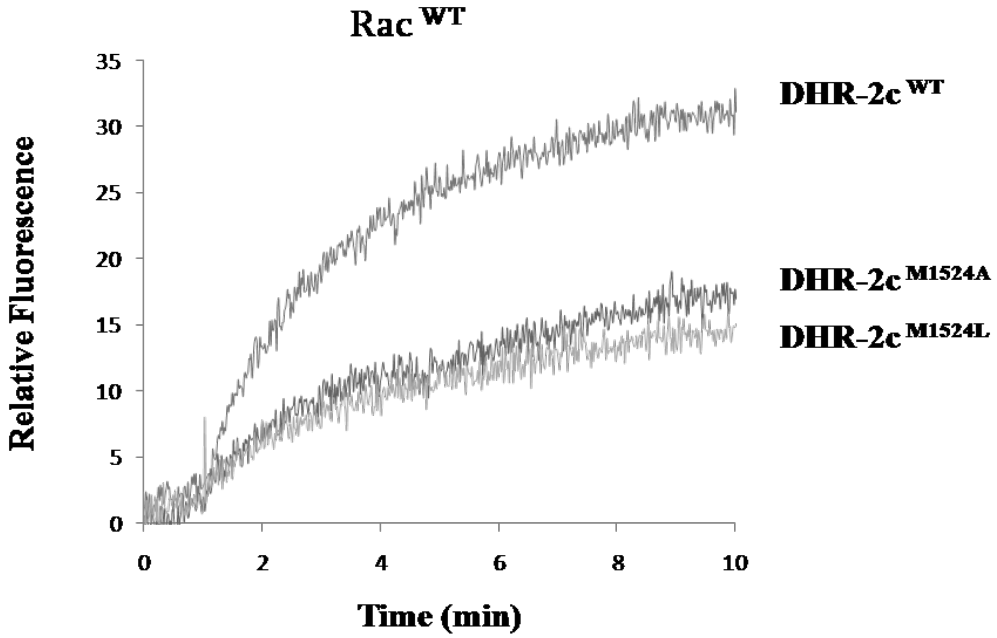

DOCK1/180
DOCK2
DOCK5
DOCK3
DOCK4
DOCK6
DOCK7
DOCK8
DOCK10
DOCK11
DOCK9
DOCK9 residues
1930
T3
1939
F56
F56
1950
1960
α10 N
α10 insert
α10 C

Figure 3.5 Met1524 of DHR-2c is critical for Rac activation. Mutation of Met1524 of DHR-2c significantly reduced its GEF activity. DHR-2c, DHR-2c (Met1524Leu), and DHR-2c (Met1524Ala) (60 nM, each) were mixed with 600 nM Rac and 1 μM mant-GDP in HMA buffer. The mant-GDP exchange rates for the mutants are about 20% of the exchange rate catalyzed by wild-type DHR-2c.


Rac WT
Relative Fluorescence
35
30
25
20
15
10
5
0
0
2
4
6
8
10
Time (min)
DHR-2c WT
DHR-2c M1524A
DHR-2c M1524L

sensitive to the presence of this methionine residue on DHR-2c. More interestingly, although the DHR-2c (Met1524Leu) mutant lost 80% of its GEF activity toward Rac, it regained the ability to stimulate nucleotide exchange on the Rac (Trp56Phe) mutant. As mentioned above, the Rac (Trp56Phe) mutant does not respond to either wild-type DHR-2c or the DHR-2c (Met1524Ala) mutant. However, the DHR-2c (Met1524Leu) mutant is quite efficient at activating the Rac (Trp56Phe) mutant (Figure 3.6). A possible explanation is that changing Trp56 to phenylalanine in Rac disrupts the interaction between Trp56 and Met1524 of DHR-2c. As shown in Figure 3.7, computer modeling predicts that Trp56 undergo a hydrophobic interaction with Met1524. If Trp56 is mutated to phenylalanine, because the phenyl ring is much smaller than the indole ring, this hydrophobic interaction may not occur. However, the substitution of Met1524 to Leucine enables an interaction between Phe56 and Leu1524 to occur because of the extra methyl group, which is similar to the leucine-phenylalanine interaction that occurs between Zizimin1 and Cdc42 (Figure 3.3). These results demonstrate that Met1524 of Dock180 and Trp56 of Rac are critical residues for the Rac-Dock180 interaction. On the other hand, the corresponding interaction between a leucine residue on Zizimin1 and a phenylalanine on Cdc42 is essential for the GEF activity exhibited by Zizimin1 toward Cdc42. Overall, these findings confirm that Trp56 of Rac is responsible for the specific recognition of Dock180 and Tiam1 by Rac.

Figure 3.6 Met1524 of DHR-2c specifically recognizes Trp56 of Rac. DHR-2c (Met1524Leu) activates the Rac (Trp56Phe) mutant. DHR-2c, DHR-2c (Met1524Leu), or DHR-2c (Met1524Ala) (600 nM each), were mixed with 600 nM Rac and 1 μM mant-GDP in HMA buffer. Only DHR-2c (Met1524Leu) showed significant GEF activity toward Rac, while both wild-type DHR-2c and DHR-2c (Met1524Ala) can not stimulate nucleotide exchange on Rac (W56F).

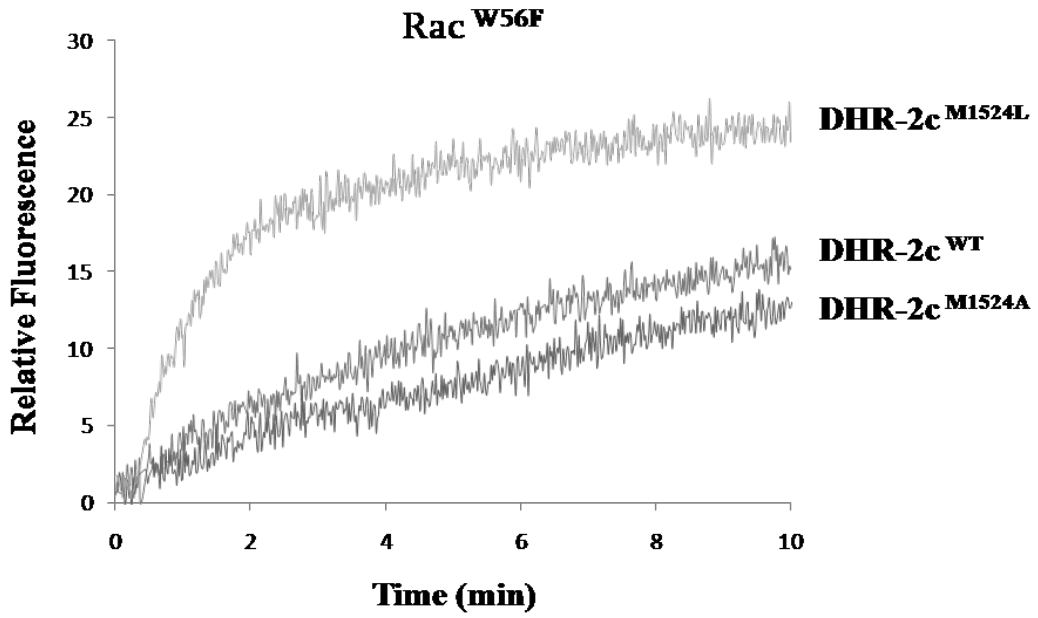
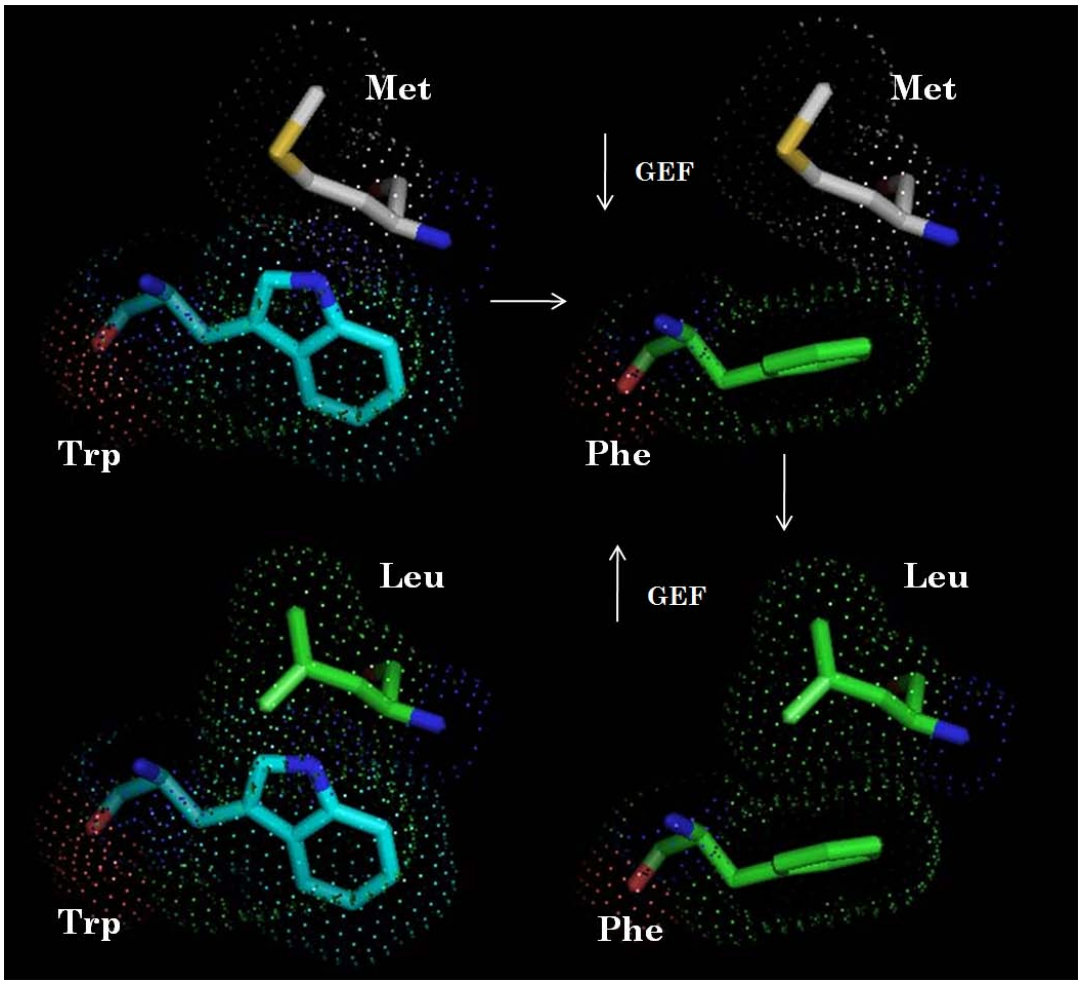

Rac W56F
Relative Fluorescence
Time (min)
0
5
10
15
20
25
30
0
2
4
6
8
10
DHR-2c M1524L
DHR-2c WT
DHR-2c M1524A

Figure 3.7 Computer prediction of the interaction between Trp56 of Rac and M1524 of DHR-2c using Pymol. The Trp-Met hydrophobic interaction in Dock180^{DHR-2c}-Rac (Phe-Leu interaction in Dock9^{DHR-2}-Cdc42) may contribute to their specific recognition to each other. Mutating Trp56 to Phe56 disrupts the hydrophobic interaction while mutating Met1524 to Leu1524 rebuilds the interaction which enables Rac(Trp56Phe) to be activated by DHR-2c (Met1524Leu) mutant.


Met
Met
GEF
Trp
Phe
Leu
Leu
GEF
Trp
Phe

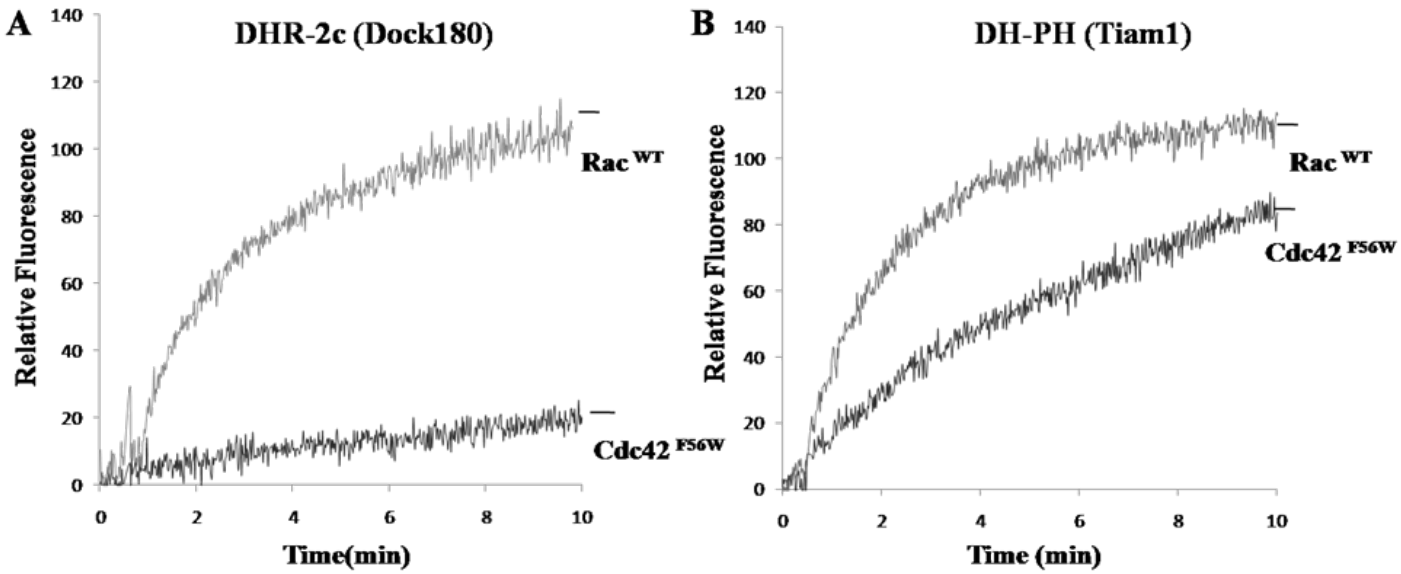
3.3.3 Trp 56 of Rac is not sufficient to specifically recognize Dock180

Trp56 of Rac and Phe56 of Cdc42 have been reported to be the residues that are responsible for the specific interaction of these GTPases with their Dbl-family GEFs. Thus, when Trp56 of Rac is changed to phenylalanine, the resultant Rac (Trp56Phe) mutant can no longer be recognized by a Rac-specific GEF, whereas it can be activated by a Cdc42-GEF of the Dbl family. The reverse is true when Phe56 of Cdc42 is changed to Trp56, i.e. Cdc42 can be activated by a Rac-GEF. We have already demonstrated Trp56 of Rac is also important for DHR-2c binding and activation. However, is this residue all that is necessary for ensuring specificity with regards to interaction with Dock180-family GEFs?

Both the Rac (Trp56Phe) and Cdc42 (Phe56Trp) mutants were prepared in order to compare their abilities to be activated by the DH-PH domain of Tiam1 and the DHR-2c domain of Dock180. Mant-GDP-exchange assays (Figure 3.2B) showed that the Rac (Trp56Phe) mutant can no longer be activated by the DH-PH domain of Tiam1. On the other hand, the Cdc42 (Phe56Trp) mutant is able to couple to the DH-PH domain of Tiam1 and exhibits a similar rate of nucleotide exchange (~80%) as wild-type Rac (Figure 3.8B).

While Trp56 of Rac is important for the specific recognition of DHR-2c, the Cdc42 (Phe56Trp) mutant is still unable to be recognized by DHR-2c. Figure 3.8A shows that the DHR-2c domain is absolutely incapable of stimulating nucleotide exchange on the Cdc42 (Phe56Trp) mutant. These results suggest that Trp56 of Rac is necessary but not sufficient for specific recognition by DHR-2c. Thus, there must be additional residues that also contribute to Dock180-GEF recognition.

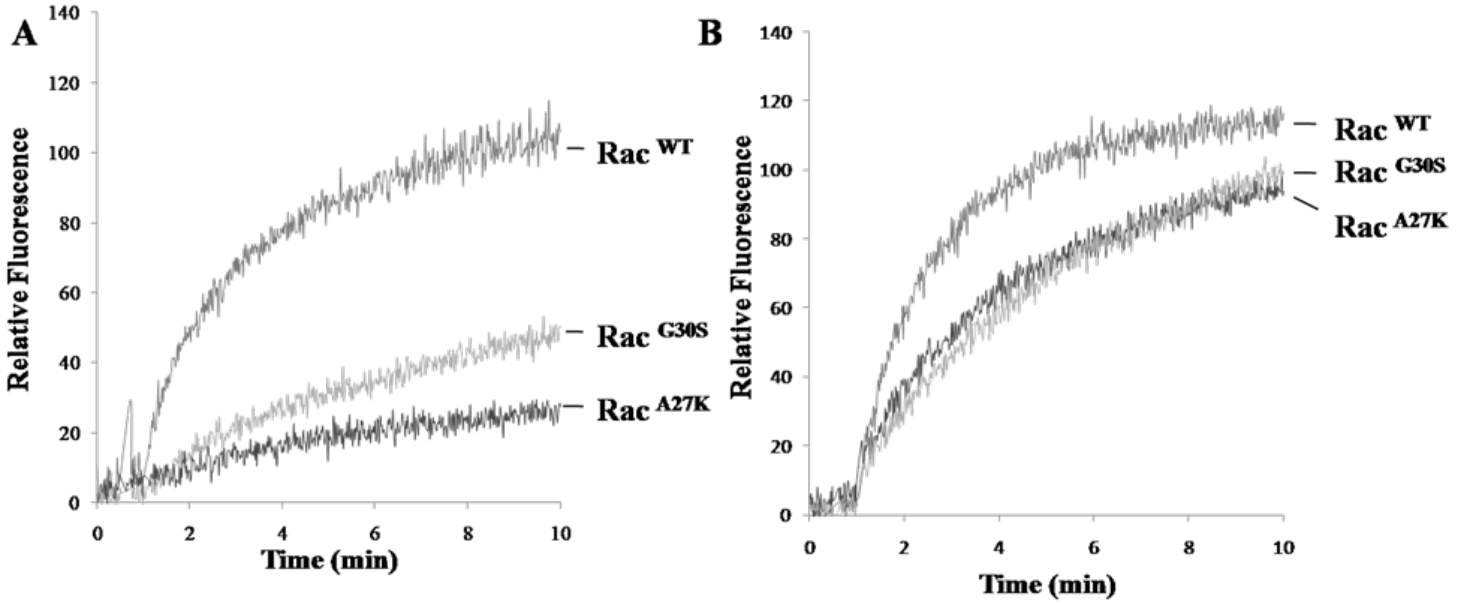
Figure 3.8 The Cdc42 (Phe56Trp) mutant can not be activated by the DHR-2c domain of Dock180 while it can be activated by the DH-PH domain of Tiam1. (A) 180 nM DHR-2c was mixed with 600 nM wild-type Rac or the Cdc42 (Phe56Trp) mutant. Nucleotide exchange can not be stimulated on the Cdc42 mutant by DHR-2c (compared to wild-type Rac). (B) 15 μM Tiam1 was mixed with 600 nM wild-type Rac or the Cdc42 (Phe56Trp) mutant. The nucleotide exchange rate for the Cdc42 mutant is similar to that for wild-type Rac stimulated by the DH-PH domain of Tiam1.


A
DHR-2c (Dock180)
Relative Fluorescence
Time(min)
Rac WT
Cdc42 F56W
B
DH-PH (Tiam1)
Relative Fluorescence
Time (min)
Rac WT
Cdc42 F56W

3.3.4 Another specific interaction is between the Switch I region of Rac and the DHR-2c domain of Dock180

Given the results described in the preceding section, we set out to search for other residues that cooperate with Trp56 in order to mediate the specific interaction with DHR-2c. We suspected that candidate residues were those that differed between Cdc42 and Rac. It has already been reported that residues downstream from position 69 on Rac and Cdc42 are not involved in GEF recognition (*5*). Therefore, wenarrowed down the candidates to Ala27, Gly30, Ser41, Ala42, Asn43, Val46, Asp47, Lys49, Val51 and Asn52 on Rac (and Lys27, Ser30, Ala41, Val42, Thr43, Ile46, Gly47, Glu49, Try51 and Thr52 on Cdc42). Based on the structure of the Zizimin1-Cdc42 complex, residues 42-51 of Cdc42 do not participate in binding to Zizimin1. Glu49 of Cdc42 is in close proximity to Arg1753 of Zizmin1. However, this arginine is also conserved in Dock180 and is not present in the DHR-2c construct. Mutagenesis work has also shown that mutating residue Ser41 or Asn52 of Rac to the cor-responding residues in Cdc42 does not affect its full activation by DHR-2c (data not show). Only Ala27 and Gly30 of Rac have been shown to be important for DHR-2 activation. We changed Ala27 of Rac to lysine and Gly30 to serine, i.e. the corresponding residues in Cdc42. These two mutants can be well expressed in bacteria and are still capable of undergoing EDTA-stimulated nucleotide exchange. Fluorescent data (Figure 3.9) showed that both mutants significantly lost their ability to be activated by DHR-2c. DHR-2c is ineffective at stimulating nucleotide exchange on Rac (Ala27Lys), just as it is ineffective with the Rac (Trp56Phe) mutant. The rate for DHR-2c-stimulated nucleotide exchange on the Rac (Gly30Ser) mutant is only 20% of that for wild-type Rac. These data showed that both Ala27 and Gly30 of Rac are critical for DHR-2c

Figure 3.9 Ala27 and Gly30 of Rac are critical for Rac activation by DHR-2c. (A) 180 nM DHR-2c was mixed with 600 nM wild-type Rac, Rac (Ala27Lys), or Rac (Gly30Ser). The Rac (Ala27Lys) mutant can not be activated DHR-2c and Rac (Gly30Ser) was activated weakly by DHR-2c (B) 15 μM Tiam1 was mixed with 600 nM wild-type Rac, Rac (Ala27Lys), or Rac (Gly30Ser). Both mutants showed similar activation by DHR-2c, as wild-type Rac.

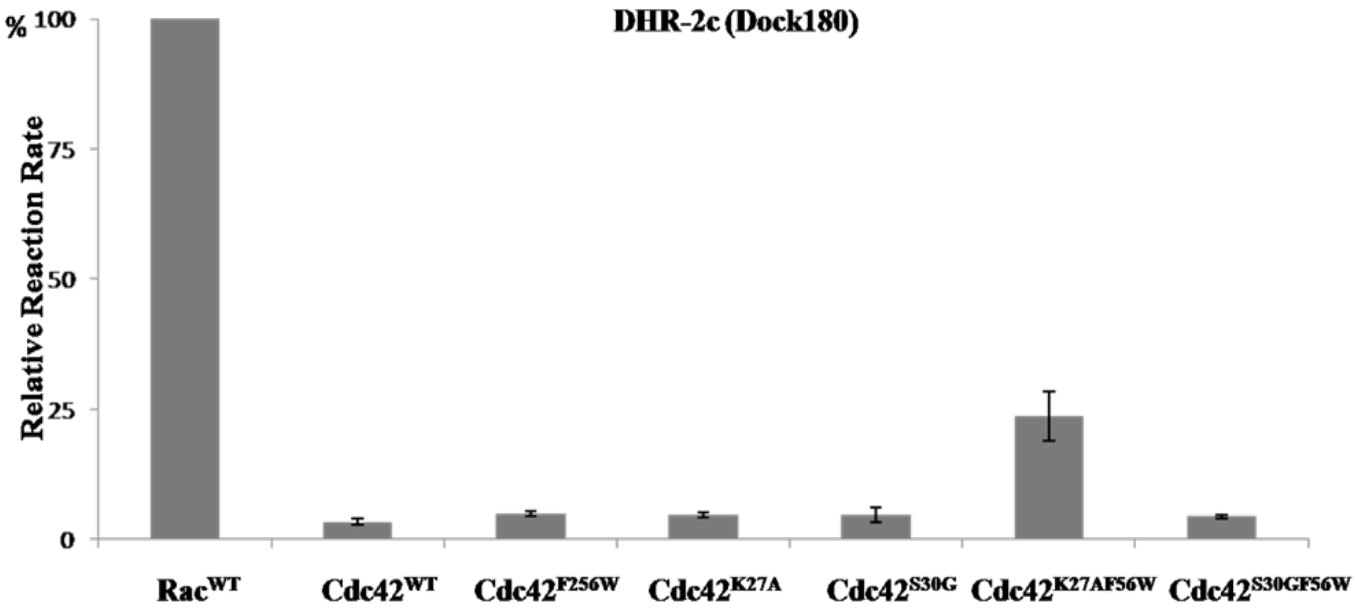

A
Relative Fluorescence
Time (min)
Rac WT
Rac G30S
Rac A27K
B
Relative Fluorescence
Time (min)
Rac WT
Rac G30S
Rac A27K

binding and the interaction between switch I of Rac and DHR-2c is important for Rac activation.

The switch I region of Rho GTPases is also an important binding region for Dbl-family GEFs, although it is not responsible for specific recognition of the GEF. Single residue substitutions in Rac or Cdc42 (i.e. Tyr 32, Asp38 or Asp39) either resulted in an inability of Rac to bind to Dbl-family GEFs or abolished the GEF-catalyzed nucleotide exchange. However, based on x-ray structure for Tiam1-Rac complex, there are no direct interactions between residue 27 or 30 of Rac and Tiam1. We tested the activation of Rac (Ala27Lys) and Rac (Gly30Ser) by the DH-PH domain of Tiam1. Figure 3.8B shows that both mutations do not affect the Rac activation by Tiam1. Ala27 and Gly30 of Rac are not important for Tiam1 binding and activation, which is different from DHR-2c.

Residue 27 and 30 are divergent residues when comparing Rac and Cdc42, and they are important for Rac activation by DHR-2. Although the Cdc42 (Phe56Trp) mutant can not be activated by DHR-2c, we were interested in seeing whether substitutions at position 27 or 30 of Cdc42, together with a substitution at position 56, yielded a Cdc42 double-mutant that can be recognized by DHR-2c. To examine this possibility, multiple mutants were prepared were prepared and expressed in *E.coli* including single mutants Cdc42 (Lys27Ala) and Cdc42 (Ser30Gly), double mutants Cdc42 (Lys27Ala-Phe56Trp) and Cdc42 (Ser30Gly-Phe56Trp), and triple mutant cdc42(Lys27Ala-Ser30Gly-Phe56Trp. Mant-nucleotide exchange assays were performed with all these mutants. As shown in Figure 3.10, All of the single mutants can't response to DHR-2c and neither the Cdc42 (Ser30Gly-Phe56Trp) double-mutant, similar to the Cdc42 (Phe56Trp) mutant. However, the Cdc42 (Lys27Ala-Phe56Trp) double-mutant was partially activated by DHR-2c.

Figure 3.10 Activation of Cdc42 mutants by DHR-2c. Single substitutions at residues 27,30 or 56 of Cdc42 do not make Cdc42 responsive to DHR-2c. Only the Cdc42 (Lys27Ala-Phe56Trp) double-mutant can be partially (~25% comparing to wild-type Rac) activated by DHR-2c. The different Cdc42 mutants (600 nM), or wild-type Rac or Cdc42 was mixed with 180 nM DHR-2c. Cdc42 (Lys27Ala-Phe56Trp) can be activated by DHR-2c while the other Cdc42 mutants are not capable of being activated by DHR-2c.


DHR-2c (Dock180)
% 100
75
50
25
0
Relative Reaction Rate
Rac^WT
Cdc42^WT
Cdc42^F256W
Cdc42^K27A
Cdc42^S30G
Cdc42^K27AF56W
Cdc42^S30GF56W

When compared with the Cdc42 (Phe56Trp) mutant which can be fully activated by the Rac-specific GEF Tiam1, the activation of Cdc42 (Lys27Ala-Phe56Trp) by DHR-2c is poor. However, this is the only Cdc42 mutant that we have found to be recognized and activated by DHR-2c. Moreover, the *kcat* for the activation of Cdc42 (Lys27Ala-Phe56Trp) by DHR-2c is still higher than the *kcat* for wild-type Cdc42 activated by Zizimin1 (*3*). Taken together, our findings suggest that residue 27 and 56 work together to specifically couple Rac/Cdc42 to their Dock180-GEFs.

3.4 Discussion

For the cases of both Dbl-family GEFs and Dock180-family GEFs, some members show strict specificity for Rac versus Cdc42, whereas some members activate both GTPases. The sequence similarity between Rac and Cdc42 is very high, thus raising the question of how Rac or Cdc42 can be specifically recognized by their GEFs. For Dbl-GEFs, it was demonstrated that residue 56 of Rac or Cdc42 is responsible for GEF specificity. Changing the tryptophan to phenylalanine on Rac makes it capable of being recognized by Cdc42-GEFs, and the converse is true when Phe56 of Cdc42 is changed to tryptophan, i.e. the Cdc42 (Phe56Trp) mutant can respond to the Dbl-family Rac-GEF Tiam1. Thus, the position 56 residue is entirely responsible for imparting specificity with regard to Cdc42 and Rac recognizing their specific Dbl-family GEFs.

How Rac/Cdc42 specifically recognizes their Dock180-GEFs is still unclear. We found that Trp56 of Rac is also critical for Dock180 binding and activation. The interaction between Trp56 of Rac and Met1424 of Dock180 plays a key role in the ability of Rac to specifically recognize Dock180. However, for Dock180 family

members, position 56 in Rac or Cdc42 is not the only residue responsible for GEF specificity. We found that Ala27 of Rac (Lys27 of Cdc42) also contributes to GEF specificity.

We have identified the residue on the DHR-2c (Met1424) that specifically interacts with Trp56 of Rac. The critical residues on DHR-2c which bind to Ala27 of Rac have still not been determined. From the x-ray structure for the Cdc42-Zizimin1 complex, there are several residues on Zizimin1 that may contact Lys 27 of Cdc42. Ser1813 and Thr1831 of Zizimin1 are both in close proximity to Lys27 of Cdc42 (~5.5 A) and these two residues are conserved in all Dock180-family Cdc42-GEFs. From our data, we can see that Gly30 of Rac is important for DHR-2 binding (Figure 3.11). However, this residue is not directly involved in specific GEF recognition. The double-mutant Cdc42 (Ser30Gly-Phe56Trp) can not be activated by DHR-2c and the triple-mutant Cdc42 (Lys27Ala-Ser30Gly-Phe56Trp) did not show any increased capability for being activated by DHR-2c compared to Cdc42 (Lys27Ala-Phe56Trp) double mutant. The Zizimin1-Cdc42 complex shows that there is an interaction between Glu1811 of Zizimin1 and Ser30 of Cdc42. This interaction may also contribute to the specific recognition of Zizimin1 by Cdc42.

Besides examining those residues that are different between Cdc42 and Rac, we also examined the possible roles of some other residues in contributing to DHR-2c activation. We changed those residues in switch I, II, and the •2-•3 regions which were reported to be important for binding to Dbl-family GEFs, as we wanted to see whether these residues also interact with DHR-2c. Residues in •3 strand and switch II region of Rac (i.e. Gly54, Trp56 and Glu61) are important for binding to both the

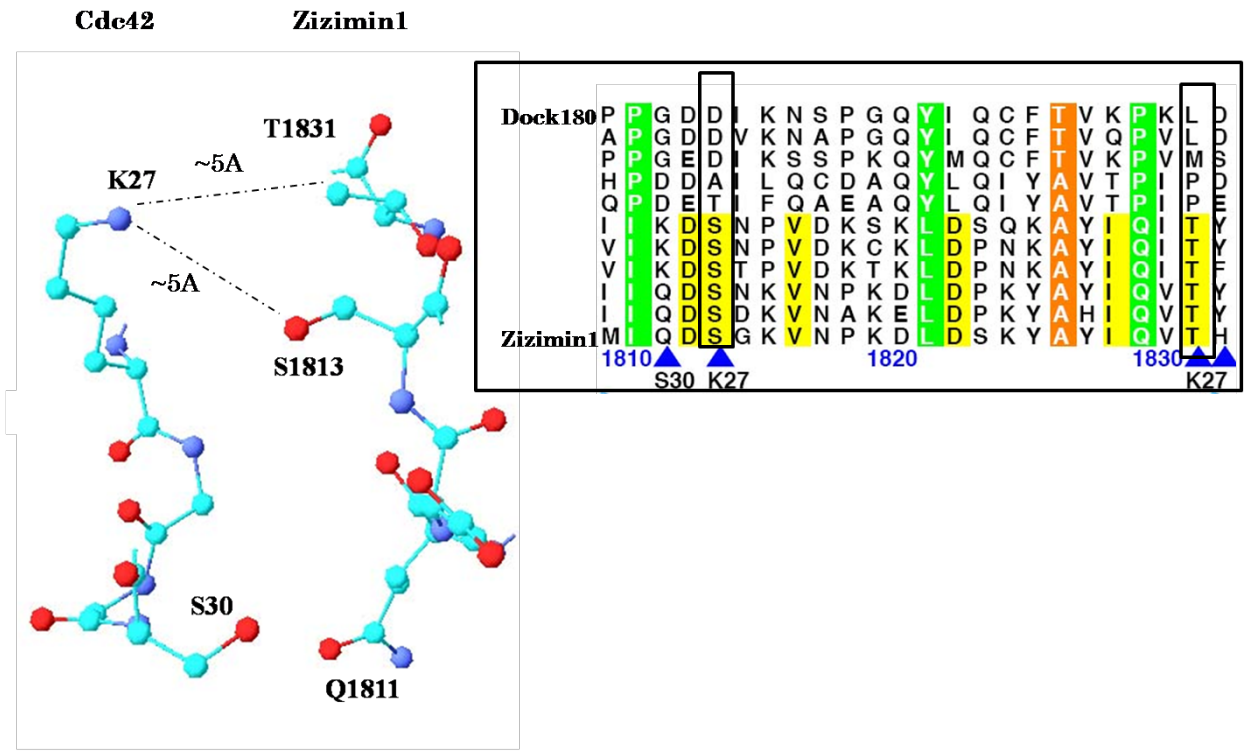

Cdc42
Zizimin1
T1831
~5A
K27
~5A
S1813
S30
Q1811
Dock180
Zizimin1
1810
1820
1830
S30
K27
K27

Figure 3.10 The contact area between Lys27/Ser 30 of Cdc42 and DHR-2c. Ser1813 and Thr1831 of Zizimin1 are both in close proximity to Lys27 of Cdc42 (~5.5 A), and Gln1811 of DHR-2c possibly interacts with Ser 30 of Rac. And both of the residues are conserved in all Cdc42 Dock180-GEFs.

DHR-2c and DH domains. In the switch I region, the critical residues which contribute to the binding of DHR-2c versus the DH domain are different. Ala27 and Gly30 of Rac respond differently to the DHR-2c and DH domains. In addition, we found that the Tyr32Ala mutant of Rac loses its sensitivity toward DH domains while it is still able to be activated by DHR-2c. Glu39Ala, another substitution at the end of switch I, is unable to be activated by both DHR-2c of Dock180 and the DH domain of Tiam1. These data show that switch I region is important for the binding of both Dock180 and Dbl-family GEFs. However, the specific binding sites are different for these two families of GEFs.

REFERENCES

1. Worthylake, D. K., Rossman, K. L., and Sondek, J. (2000) Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1, *Nature 408*, 682-688.
2. Gao, Y., Xing, J., Streuli, M., Leto, T. L., and Zheng, Y. (2001) Trp(56) of rac1 specifies interaction with a subset of guanine nucleotide exchange factors, *J Biol Chem 276*, 47530-47541.
3. Yang, J., Zhang, Z., Roe, S. M., Marshall, C. J., and Barford, D. (2009) Activation of Rho GTPases by DOCK exchange factors is mediated by a nucleotide sensor, *Science 325*, 1398-1402.
4. Gao, Y., Dickerson, J. B., Guo, F., Zheng, J., and Zheng, Y. (2004) Rational design and characterization of a Rac GTPase-specific small molecule inhibitor, *Proc Natl Acad Sci U S A 101*, 7618-7623.
5. Kwofie, M. A., and Skowronski, J. (2008) Specific recognition of Rac2 and Cdc42 by DOCK2 and DOCK9 guanine nucleotide exchange factors, *J Biol Chem 283*, 3088-3096.

CHAPTER 4

CONCLUSION

Rho GTPases participate in a wide range of cellular activities including cell mobility, cytoskeletal organization and activation of specific kinases. Rho family proteins exist in two different states, a signaling-inactive GDP-bound and a signaling-active GTP- bound state. In order to switch between these two states, GEFs help stimulate the exchange of GDP for GTP on Rho GTPases.

Two families of Rho GEFs have already been discovered. The classical Dbl-family of GEFs consists of more than 60 members in humans with each member qsharing a conserved tandem DH-PH domain (*1*). The newly discovered Dock180-GEF family consists of 11 members in humans (*2*). They share very low sequence similarity with the Dbl-family and instead share two conserved domains designated as the DHR-1 and DHR-2 domains. Within the 11 family members, Dock180 is best studied.

Various studies have shown that Dock180 and its homologues in *Drosophila* (Myoblast City) and *C. elegans* (Ced-5) are involved in a number of biological processes including actin cytoskeleton reorganization, phagocytosis, cell migration and myoblast fusion (*3-5*). Dock180 was shown to be an upstream regulator of Rac and biochemical analysis confirmed that it functioned as a Rac-GEF (*6*). The DHR-2 domain of Dock180 was demonstrated to be responsible for its activity.

In my thesis work, I was focusing on the biochemical characterization of the limit domain (DHR-2c) of Dock180 and investigation of the activation mechanism on Rac. The main finding from my thesis research are listed below.

1. DHR-2c is a limit sub-domain in Dock180 which functions as a specific GEF for Rac. The DHR-2 domain of Dock180 in *E.coli* is not well behaved in low salt solution, making it difficult to purify this functional domain to homogeneity and to reliably measure its guanine nucleotide exchange activity. Based on secondary structure analysis and homology modeling of the DHR-2 sequence, together with the recently published structure of the Dock-9/Cdc42 complex (*7*), I delineated a limit-functional domain for Dock180 (residues 1335-1657) that binds its cognate GTPase Rac in a manner typical of a GEF. In addition, the ~37 kDa DHR-2c domain showed robust activity toward purified Rac in fluorescence-based GEF assays, whereas I found no evidence that the amno-terminal half of the DHR-2 domain (residues 1178-1334) contributes to the overall GEF activity of the DHR-2 domain. *In vivo* experiments have also confirmed that the DHR-2c domain can activate Rac in cells.

2. DHR-2c shows high GEF activity compared to other GEFs. In order to examine the catalytic potential of the DHR-2c domain, I performed GEF assays under conditions of excess Rac GTPase. Different concentrations of purified mant-GDP-Rac were mixed with excess GDP and sub-stoichiometric amounts of DHR-2c. Using this readout, I estimated a k_{cat} value of 19.8/min (0.33/sec) for DHR-2c which is 1000-fold faster than the intrinsic GDP-dissociation rate constant for Rac. It is also much higher than the activity of most of the Dbl-Rac GEFs and other Dock180 family members.

3. The Trp56 of Rac specifically recognizes the Met1524 of DHR-2c. In order to explore the factors underlying the relative selectivity of the GEF activity of DHR-2c towards Rac/Cdc42, I evaluated the importance of Trp 56 of Rac. I found that the Rac (Trp56Phe) mutant is activated to <10% of the level of wild-type Rac by DHR-2c. Using the Cdc42-Dock9 x-ray crystal structure as a guide (*7*), I determined that the methyl group of Leu1940 in Dock9 interacts with the phenyl side-chain of Phe. This leucine residue is conserved in members of the Dock subfamily D (Dock 9-11

which are specific for Cdc42), while in the Rac-specific subfamily A (Dock 1,2, and 5; Dock1 being Dock180), the corresponding residue is methionine. I mutated the corresponding residue Met1524 to leucine in DHR-2c and found the removal of the side-chain of methionine decreases the GEF activity of DHR-2c by ~80%. Conversely, the leucine substitution resulted in a five-fold increase in GEF toward the Rac (Trp56Phe) mutant. Taken together, these results provided insight into how Dock180 versus Zizimin1 discriminate between Rac versus Cdc42.

4. Ala 27 and Trp 56 of Rac both contribute to the specific recognition for Dock180-family Rac GEFs. Tryptophan 56 of Rac is necessary for DHR-2c binding and activation, similar to what has been observed for the DH-domain of Tiam1. However, the complementary substitution (i.e. Phe to Trp) at position 56 in Cdc42 is not sufficient to allow for nucleotide exchange catalyzed by DHR-2c which indicates that additional contacts are necessary for full GEF activity. Further mutagenesis experiments showed that Ala 27 of Rac is also important for specific recognition by Dock180. Fluorescence GEF assays demonstrated the Rac (Ala27Lys) mutant was severely impaired in its ability to functionally couple to DHR-2c while the Cdc42 (Ser30Gly-Phe56Trp) double-mutant showed a partial ability to recognize DHR-2c. Thus far, I have not found any other residue that contributes to the specific recognition of DHR-2c. Taken together, I conclude that the •3 and switch II region of Rho GTPases are the critical areas for the binding of both Dock180-family and Dbl-family GEFs. The switch I region in the GTPases also plays an important role in the specific binding of both Dock180 and Dbl-family GEFs but significantly, the residues which make up the contact residues are different for these two families of GEFs.

Future investigation

The results from my work on the characterization of the DHR-2c domain of Dock180 suggest two future areas for investigation. Although it is now clear that the DHR-2c domain of Dock180 can activate Rac both *in vitro* and *in vivo* at high efficiency, it is not entirely understood how this GEF domain works on Rac. While the x-ray structure of the Zizimin1-Cdc42 complex is already available, because of the low primary sequence similarity between Zizimin1 and Dock180, it is very hard to predict the detailed mechanism of the Dock180-mediated Rac activation. An exciting but difficult challenge that remains is the determination of an x-ray crystal structure of the DHR-2 or the DHR-2c domain. I have already put a great deal of effort intp crystallization trials (see appendix) and made some improvements. However, a diffractable crystal has still not been obtained. The next step will be to try and generate new constructs of the DHR-2c domain based on secondary structural predictions and the x-ray crystal structure of the Zizimin-Cdc42 complex. Attempts should be make toward moving the flexible region of the C-terminal of DHR-2c or adding a helical region at the N-terminus to match the Zizimin1^{DHR-2} construct.

Future efforts should also be invested in additional mutagenesis work on both DHR-2c and Rac. I have already shown that Met1524 of Dock180 specifically interacts with Trp56 of Rac. There is another important residue, Asn1527 in DHR-2c, which also makes hydrogen bonding constant with Trp56 of Rac. This residue is also divergent between Dock180 and Zizimin1 (i.e. the latter has a glutamine residue at the same position). It would be of interest to mutate this residue and check to see whether it also contributes to Rac-specific recognition. In addition, there are several residues within Dock180 which are in close distance to Ala27 of Rac, another critical residue that helps Rac to couple to DHR-2c. Finally, in the future it would be interesting to

prepare recombinant Zizimin1 and determine whether these residues that have been to be important for the Dock180-mediated activation of Rac play corresponding roles in the ability of Zizimin1 to activate Cdc42. Collectively, the results of these experiments would go a long way toward providing a comprehensive picture of how Dock180-family GEFs activate their target GTPases.

REFERENCES

1. Erickson, J. W., and Cerione, R. A. (2004) Structural elements, mechanism, and evolutionary convergence of Rho protein-guanine nucleotide exchange factor complexes, *Biochemistry 43*, 837-842.
2. Cote, J. F., and Vuori, K. (2002) Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity, *J Cell Sci 115*, 4901-4913.
3. Erickson, M. R., Galletta, B. J., and Abmayr, S. M. (1997) Drosophila myoblast city encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization, *J Cell Biol 138*, 589-603.
4. Wu, Y. C., and Horvitz, H. R. (1998) C. elegans phagocytosis and cell-migration protein CED-5 is similar to human DOCK180, *Nature 392*, 501-504.
5. Reddien, P. W., and Horvitz, H. R. (2000) CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in Caenorhabditis elegans, *Nat Cell Biol 2*, 131-136.
6. Brugnera, E., Haney, L., Grimsley, C., Lu, M., Walk, S. F., Tosello-Trampont, A. C., Macara, I. G., Madhani, H., Fink, G. R., and Ravichandran, K. S. (2002) Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex, *Nat Cell Biol 4*, 574-582.
7. Yang, J., Zhang, Z., Roe, S. M., Marshall, C. J., and Barford, D. (2009) Activation of Rho GTPases by DOCK exchange factors is mediated by a nucleotide sensor, *Science 325*, 1398-1402.

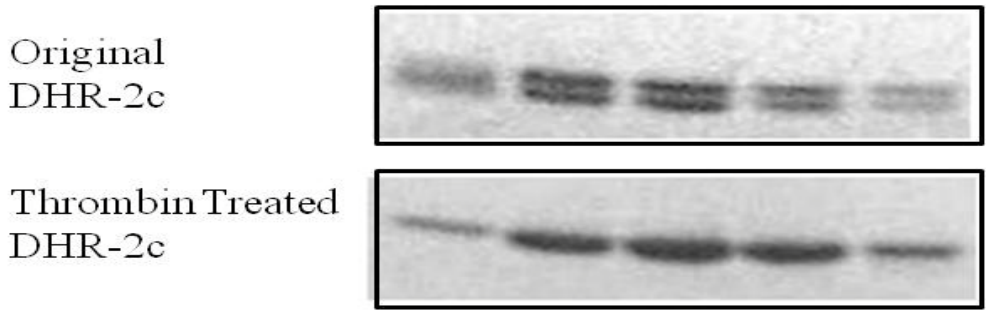
APPENDIX

Purification of DHR-2c and the DHR-2c-Rac complex

Since x-ray the structure for the complex between Tiam1 and Rac was solved in 1998 (*1*), a number of structures of Dbl-GEFs and their complexes with Rho GTPases have been determined either by x-ray crystallography or NMR spectroscopy (*2*). Based on this molecular information, a good deal of work has been done to investigate the detailed catalytic mechanism of Dbl-GEFs. Only recently has structural information become available for a Dock180-family member based on the reported x-ray structure of DHR-2 (Dock9) bound to Cdc42 (*3*). However, because of the low similarity between Zizimin1 and Dock180, and given that many experimental results obtained for the Dock180-catalyzed activation of Rac can not be explained by analyzing the structure of the Zizimin1-Cdc42 complex, it is still of great interest to obtain structural information for a Dock180-Rac complex. Full-length Dock180 is approximately 180 kDa and is difficult to express in bacteria. Likewise the DHR-2 domain (amino acids 1111 to 1657) is difficult and a slightly shorter version of the DHR-2 domain (amino acids 1178 to 1657) is not stable in low salt solution and tends to aggregate. Thus, we are putting significant effort into the newly delineated DHR-2c sub-domain. This functional sub-domain can be expressed in *E.coli* and is stable in low salt solution. If we can purify the DHR-2c sub-domain or the complex of DHR-2c and Rac, it should be possible to crystallize the proteins and solve their structures.

DHR-2c is stable in solution with 100 mM NaCl. However, after purification, the DHR-2c preparation appeared as a doublet on SDS PAGE (Appendix-1). The two bands were confirmed to be DHR-2c by Western blot analysis and further purification did not resolve the two bands. After I excluded the possibility of degradation, I

Appendix-1 DHR-2c is a doublet on SDS-PAGE (7%-15% gradient gel). This is likely due to the partial degradation of the His-Tag. Thrombin treatment removed the His-Tag and yield a single protein band.


Original
DHR-2c
Thrombin Treated
DHR-2c

suspected that the doublet was due to the partial degradation of the His-tag. To confirm this, I treated DHR-2c with thrombin, following the nickel affinity column, at 4°C overnight. SDS PAGE showed that DHR-2c was uniform and appeared as a band (Appendix-1). A final gel-filtration step yielded a preparation of DHR-2c that was greater than 95% pure.

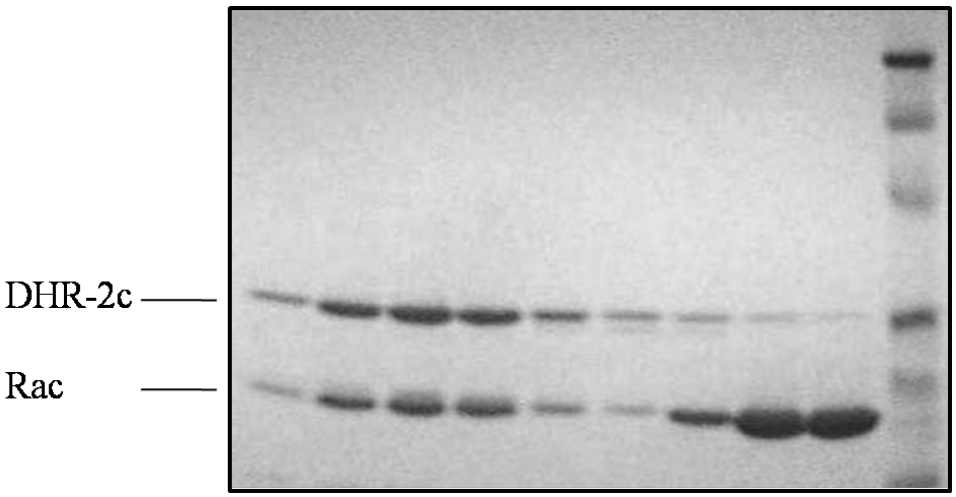
The purification of Rac was straight-forward. The critical element in the purification is Mg^{2+}, which is needed throughout the entire purification process. Without $MgCl_2$, Rac can not bind GDP with high affinity and degrades easily. In order to form a stable complex, DHR-2c and Rac were mixed in a molar ratio 1:2 in buffer with EDTA (see Chapter 2 "Methods"). A G75-sizing column was used to separate the Rac-DHR-2c compelx from free Rac protein (Appendix-2).

Crystallization of DHR-2c and the DHR-2c-Rac complex

Our ultimate aim is to crystallize DHR-2c, alone, and bound to Rac. I have made some progress on these efforts which are still ongoing. When I set up crystallization screens for DHR-2c, I found out that it was easy to precipitate at relatively low concentrations (3-5 mg/ml). Although DHR-2c is much more stable than DHR-2, soluble DHR-2c still exhibited a monomeric state and an aggregation state during purification process as shown in Figure 2.11. Although I collected the proteins in the monomeric state, I suspected that a portion of the proteins aggregated when their concentration was increased. This may prevent DHR-2c from crystallizing.

The complex between DHR-2c and Rac appears to be reasonably stable (Appendix-2). As mentioned above, DHR-2c prevents Rac from undergoing degradation. There is a possibility that Rac also prevents DHR-2c from self-

Appendix-2 Isolation of a complex between DHR-2c and Rac. DHR-2c and Rac were mixed at a 2:1 molar ration and applied to a Superdex G75 gel-filtration column. The first protein peak represents a complex of DHR-2 and Rac, while the second peak represents excess (free) Rac.


DHR-2c
Rac

aggregation because the complex between Rac and DHR-2c can be concentrated to a maximum concentration of 100 mg/ml. Macro Screen 1-2, Index Screen 1-2, and all other screening conditions from the Hampton Research Kit were used to screen for the proper crystallization condition for the complex. Thus far, I found some conditions which gave micro-crystals of the complex. However, well-organized or diffractable crystals have not yet been obtained. Additional screens are still in process to obtain good-quality crystals. I am also trying to add some reagents (glycerol, NDSB-201) which will prevent protein aggregation and were used for the crystallization of the Dock9-Cdc42 complex. Modification of the DHR-2c domain, such as adding more residues at the N-terminus, is also under consideration.

REFERENCES

1. Worthylake, D. K., Rossman, K. L., and Sondek, J. (2000) Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1, *Nature 408*, 682-688.
2. Worthylake, D. K., Rossman, K. L., and Sondek, J. (2004) Crystal structure of the DH/PH fragment of Dbs without bound GTPase, *Structure 12*, 1078-1086.
3. Yang, J., Zhang, Z., Roe, S. M., Marshall, C. J., and Barford, D. (2009) Activation of Rho GTPases by DOCK exchange factors is mediated by a nucleotide sensor, *Science 325*, 1398-1402.